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Účinky reovírusu na nádorové bunky

Effects of reovirus on cancer cells

Dizertačná práca

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1. Zoznam použitých skratiek a symbolov

BNIP3 – BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, člen BCL2/adenovírus E1B 19 kd-interagujúcej proteínovej (BNIP) rodiny, proapoototický proteín

BNIP3L (NIX) BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like, funkčný homológ BNIP3

CD4 – glykoproteín na povrchu imunitných buniek, člen imunoglobulínovej superrodin, znak T_H lymfocytov

CD8 – transmembránový glykoproteín, ko-receptor pre receptor T buniek (TCR), špecifický pre MHC I proteíny, znak T_c lymfocytov

dsRNA – dvojvláknová ribonukleová kyselina

EGFR – epidermal growth factor receptor

eIF2α – alfa podjednotka iniciačného faktoru 2

G207 – modifikovaný onkolytický herpes simplex vírus

GEF – guanin exchange factor

GLUT1 – glucose transporter 1, uľahčuje transport glukózy cez plazmatickú membránu

GTP/ GDP - Guanouín-5'-trifosfát/difosfát, substrát syntézy RNA/defosforylačný produkt GTP

HIF – hypoxiou indukovaný faktor

HSV – herpes simplex vírus

IGF II – insulin like growth factor 2, hlavný fetálny rastový faktor

ISVP – intermediárna subvírusová častica

JNK – c-Jun N-terminal kináza, patrí do rodiny mitogénom aktivovaných proteín kináz, odpoveď na stres

MEFs – myšacie embryonálne fibroblasty

Met-tRNA – transferová RNA s naviaznaným metionínom

MK16 – imortalizované bunky myších obličiek

MOI – multiplicity of infection = pomer infekčných viriónov k počtu napadnutých buniek

mRNA – messenger RNA

MV-Edm – Edmonston B druh osýpkového vírusu

NDV – Newcastle disease virus

NIH 3T3 – štandardná myšia fibroblastová bunková línia

ONYX15 – deletovaný adenovírus

p53 – tumor supresorový proteín s molekulovou hmotnosťou 53 kDa, tzv. strážca genómu

PFU – plaque forming units = miera počtu častíc schopných tvoriť plaky v jednotke objemu, funkčná jednotka

PHD – prolyl hydroxylačné enzýmy

PI3 kináza - fosphatidylinozitol 3-kináza

PKR – dvojvláknovou RNA aktivovaná proteínkináza

RNA – rionukleová kyselina

SH2 doména – štrukturálne konzervovaná proteínová doména v Src onkoproteíne

Src – protoonkogén, rodina ne-receptorových tyrozín kináz

TC-1 – imortalizované bunky myšieho pľúcneho tkaniva

TNF – tumor nekrotizujúci faktor

VEGF - Vascular endothelial growth factor, signálny proteín

VHL – von Hippel Lindau faktor

VSV – vírus vezikulárnej stomatitídy

2. Úvod

2.1 ONKOLYTICKÉ VÍRUSY

2.1.1 VÍRUSY – NOVÝ LIEK PROTI RAKOVINE

Použitie replikačne-kompetentných vírusov ako onkolytických agens prispelo k mnohým zaujímavým objavom v posledných 2 desaťročiach výskumu nádorových ochorení. A výskum zas viedol k objavu značného počtu onkolytických vírusov. Niektoré prirodzene sa vyskytujúce vírusy preferenčne infikujú a zabíjajú transformované bunky, napríklad vírus vezikulárnej stomatitídy (VSV), herpex simplex vírus (HSV), Newcastle disease virus (NDV) a reovírus (Farassati F. et al., 2001, Norman K.L. a Lee P.W., 2000, Sinkovics J.G. a Horvath J.C., 2000, Stojdl D.F. et al., 2000). Edmonston B druh osýpkového vírusu (MV-Edm) pôvodne atenuovaný pre použitie ako živá vírusová vakcína, má potenciálne onkolytické využitie (Grote D. et al., 2001). Okrem toho, niekoľko genetických modifikácií adenovírusov, herpetického vírusu a vírusu chrípky, im dokázalo vniesť selektívnu onkolytickú aktivitu (Bergmann M. et al., 2001, Dobbelstein M., 2004, Martuza R.L., 2000). Mnoho nových štúdií demonštruje, že tieto vírusy môžu efektívne a selektívne ničiť transformované bunkové línie a taktiež vykazovať protinádorové vlastnosti *in vivo*. Takisto sa enormne posunulo vpred poznanie molekulárnych základov vírusovej onkolýzy.

Onkolytické vírusy dokážu infikovať nádorové bunky, replikovať sa v nich za využitia bunkového replikačného aparátu, indukovať smrť infikovanej nádorovej bunky, uvoľňovať virálne častice schopné i neschopné ďalšej replikácie. Po rozpade napadnutej bunky sa vírus šíri do okolitého tkaniva a zosilňuje tak svoj liečebný efekt. Vírusy sa množia geometrickou radou, čo eventuálne vedie k maximalizácii dávky v žiadanom mieste účinku, kým neúplná replikácia v zdravom tkanive povedie k úspešnému odstráneniu alebo aspoň redukovaniu toxicity. Vďaka selektivite ich replikácie pre nádorové tkanivo môže byť terapeutický index takéhoto liečiva významne zvýšený. Navyše, onkolytické vírusy napomáhajú deštrukcii nádorových buniek viacerými mechanizmami. Okrem priamej lýzy, výsledku vírusového replikačného cyklu, vírusy taktiež sprostredkúvajú deštrukciu nádorovej bunky indukciou nešpecifickej i špecifickej antitumorálnej imunity. Niektoré vírusy exprimujú proteíny, ktoré sú pre nádorové bunky

cytotoxické (napr. adenovírus exprimuje cytotoxické proteíny E3 a E4ORF4) (Mullen J.T. a Tanabe K.K., 2003).

2.1.2 HISTÓRIA

Dôkazy onkolytickej aktivity pripísanej rozmnožujúcim sa vírusom boli prvýkrát popísané v kazuistikách publikovaných už roku 1912. Tieto správy opisovali raritnú, avšak dramatickú odpoveď u pacientov s nádorovým ochorením, ktorí zároveň prekonávali vírusovú infekciu (Bousser J. a Zittoun R., 1965, Hansen R.M. a Libnoch J.A., 1978, Weintraub L.R., 1969). Založené na týchto pozorovaniach boli vírusy s malou patogenicitou (minimálnou toxicitou pre normálne tkanivo) a vysokou onkolytickou kapacitou (toxické pre rakovinové bunky) vybrané pre počiatočný klinický výskum (Asada T., 1974, PACK G.T., 1950). Štúdie v päťdesiatych rokoch minulého storočia s West Nile vírusom Egypt 101 odhalili jeho protinádorovú aktivitu, ktorá sa prejavovala ako prechodná nekróza nádorov u pacientov. Buhožiaľ, ďalšie štúdie boli zastavené, keď sa u imunosuprimovaných pacientov po liečbe týmto vírusom vyvinula encefalitída (HUEBNER R.J. et al., 1956, Russell S.J., 1994, SOUTHAM C.M. a MOORE A.E., 1952). Neskôr bol testovaný atenuovaný mumps vírus a jeho onkolytické účinky boli ešte pôsobivejšie. Odpovede na liečbu však neboli trvalé a boli limitované na miesto vpichu (Asada T., 1974, Csatary L. a Gergely P., 1990, Csatary L.K. et al., 1993, Okuno Y. et al., 1978, Shimizu Y. et al., 1988, Yamanishi K. et al., 1970). Aby bolo dosiahnutého rozsiahlejšieho systémového výsledku po lokálnom injekčnom spôsobe podania, prišli na rad metódy stimulácie imunitného systému použitím vírusových „onkolyzátov“. Výskumníci exponovali malígne bunky vírusu *ex vivo*, indukovali onkolýzu a takto poskytovali antigénny materál, ktorý mohol byť použitý ako vakcína. Chrípkové vírusové „onkolyzáty“ indukovali silnejšiu protinádorovú aktivitu *in vivo* než samotný vírus alebo nemodifikované extrakty z nádorových buniek (Boone C.W. et al., 1974, Heicappell R. et al., 1986). V jednej štúdii s pacientkami s pokročilými nádormi ovárií podskupina pacientiek vykazovala trvalú odpoveď, ktorá sa zdala byť posilnená následnou liečbou interleukínom-2 (IL-2) (Ioannides C.G. et al., 1990). Ďalšie štúdie používali onkolyzáty s Newcastle disease vírusom (NDV) a preukázali signifikantné zlepšenie prežitia u pacientov vo včasných štádiách melanómu porovnaných s historickými kontrolami

(69% desaťročné prežitie bez ochorenia *versus* 15% pri kontrolách) (Lin E. a Nemunaitis J., 2004). V dnešnej dobe sa modifikovaný Newcastle disease virus (73T) sa používa ako vakcinačný onkolyzát v bežiacej klinickej štúdii zahŕňajúcej pacientov s melanómom a pacientov s karcinómom obličiek. Predbežné výsledky sa zdajú byť nádejné (Sinkovics J.G. a Horvath J.C., 2003).

2.1.3 VÍRUSY A IMUNITA

Vírusová infekcia buniek vyvoláva imunitnú odpoveď organizmu, ktorá pozostáva z možstva cytokínov (interferón α, β a γ, TNF α a niektoré interleukíny) a infiltrácie makrofágmi, neutrofilmi a NK bunkami. Pretože aktivácia klasickej cesty komplementu v nádorovej bunke nie je jediný spôsob jej zabitia, vznik skríženej rezistencie so štandardnými chemoterapeutikami alebo rádioterapiou je veľmi málo pravdepodobný. Na druhej strane, účinná imunitná odpoveď je schopná zničiť replikujúce sa virióny a takto limitovať ich priamy lytický efekt (Smith E.R. a Chiocca E.A., 2000). Toda et al. (Toda M. et al., 1999) ukázali, že liečba nádorov myší geneticky modifikovaným onkolytickým herpes vírusom G207 viedla k spusteniu systémovej imunity aj proti ďalším nádorom, v ktorých vírus nebol detegovaný, a to cytotoxickou odpoveďou T buniek. Imunosupresia navodená kortikosteroidmi v transplantovanom ľudskom nádore znižovala účinnosť G207 (Todo T. et al., 1999). Na druhej strane, Hirasawa et al. (Hirasawa K. et al., 2003) dokázali zvýšenú účinnosť reovírusu na eradikácii myších nádorov po pridaní cyklosporínu A a anti CD4 a anti CD8 protilátok. Stále však ostáva doriešiť, ktoré mechanizmy sa a v akej miere podieľajú na vzniku antivírusovej, pri terapii onkolytickými vírusmi nežiaducej, a ktoré na vzniku protinádorovej imunity.

2.1.4 IDEÁLNY ONKOLYTICKÝ VÍRUS

Aby sa na vírus mohlo nahliadať ako na zástupcu onkolytickej viroterapie, musí byť preskúmaná väčšina jeho účinnostných, bezpečtnostných a výrobných charakteristík. Vírus by sa mal replikovať v nádorových bunkách a ničiť ich, zatiaľ čo cytotoxický efekt na okolité nenádorové tkanivo obklopujúce tumor by mal byť čo najmenší. V ideálnom prípade by aj vírus podaný systémovo napádal výhradne nádorové bunky. Takisto by sa optimálny vírus množil rovnako rýchlo tak v replikujúcich sa, ako aj v nádorových bunkách v pokojovej fáze bunkového

cyklu, a to s vysokým stupňom výťažku. Rozšíril by sa celou masou tumoru buď ničiac bunky priamo lýzou, alebo by ich senzitivizoval na pôsobenie ďalších terapeuticky použitých látok, zatiaľ čo by ostával neškodný pre okolité normálne zdravé tkanivo. Výhodou by bolo, ak by rodičovský vírus spôsoboval iba jemné, veľmi dobre charakterizované a zvládnuteľné ochorenie/a. Alternatívou by bolo využitie delečných mutantov, ktoré sú sami osebe nevirulentné. Genetická stabilita vírusu je jeho veľkou prednosťou z bezpečnostného i výrobného hľadiska. Ideálny vírus by takisto bol schopný replikácie za prítomnosti vznikajúcej, alebo už pre-existujúcej imunitnej odpovede. Toto môže vyžadovať expresiu aj takých virálnych proteínov, ktoré sú zahrnuté v supresii antivirálnej imunitnej odpovede. Pretože by nastolil len minimálnu imunologickú reakciu, bol by veľmi dobre tolerovaný pacientami. Navyše, infekcia vírusom by stimulovala efektívnu antitumorálnu imunitnú odpoveď, ktorá by viedla k deštrukcii metastáz (Ring C.J., 2002). Tieto ideálne podmienky však väčšina onkolytických vírusov spĺňa iba sčasti.

2.1.5 ROZDELENIE ONKOLYTICKÝCH VÍRUSOV

Vírusy svojou infekciou dramaticky menia fenotyp napadnutej bunky práve kvôli maximalizácii vlastnej replikácie a tým prežitia. Zmeny v bunke indukované vírusovou infekciou sú často mimoriadne podobné bunkovým zmenám získaným počas karcinogenézy. (napr. inaktivácia tumor supresorového proteínu p53, inhibícia apoptózy). Preto nie je prekvapivé, že mnoho vírusov prirodzene rastie v nádorových bunkách a/alebo sa dajú vytvoriť vírusy s nádorovou selektivitou. Bolo popísaných päť základných mechanizmov, ako dosiahnuť nádorovú selektivitu vírusu.

Tieto zahŕňajú: (a) použitie vírusov s vrodenou selektivitou pre nádorovú bunku (napr. vírus newcastelskej choroby, vírus vezikulárnej stomatitídy, autonómne parvovírusy, niektoré druhy vírusu osýpok, reovírus (Alemany R. et al., 2000, Coffey M.C. et al., 1998, Kirn D.H., 2000, Lorence R.M. et al., 1988, Rommelaere J. a Cornelis J.J., 1991, Stojdl D.F. et al., 2000, Wickham T.J. et al., 1997); (b) deletovanie celých génov vírusu (napr. HSV, adenovírus, vírus vakcínie (Bischoff J.R. et al., 1996, Heise C. et al., 1997, Martuza R.L. et al., 1991, Mastrangelo M.J. et al., 2000); alebo (c) funkčných oblastí génov (adenovírus, poliovírus (Fueyo J. et al., 2000, Gromeier M. et al.,

2000, Heise C. et al., 2000), ktoré sú dôležité pre efektívnu replikáciu a/alebo toxicitu v normálnych bunkách, ktorých prítomnosť však pre replikáciu a/alebo lýzu nádorových buniek nie je nevyhnutná; (d) vnesením nádorovo/tkanivovo-špecifických promótorov do vírusov, ktoré limitujú expresiu génov potrebných na replikáciu výhradne na nádorové bunky (adenovírus, HSV) (Miyatake S. et al., 1997) alebo (e) modifikovaním vírusového obalu tak, aby bol vírus rozpoznávaný a pohlcovaný iba nádorovou bunkou (napr. adenovírus, poliovírus (Alemany R. et al., 2000, Wickham T.J. et al., 1997). Každý z uvedených postupov má samozrejme výhody i nevýhody.

Všetky z týchto vírusov preukázali selektivitu pre nádorové bunky *in vitro* a/alebo *in vivo*, pričom mnohé z nich boli podávané intratumorálne, intraperitoneálne a/alebo intravenózne. Prehľad najdôležitejších onkolytických vírusov uvádza **Tabuľka 1.**

Rodina vírusov	Onkolytický vírus	Špecificita	Genetická modifikácia
Adenovírus	ONYX-015	Bunky bez funkčného p53	E1B-55kD, E3b delécia
	CV 706	Bunky prostaty	E1A expresia riadená PSE, delécia E3
	CV 787	Bunky prostaty	E1B expresia riadená PSE
	Ad5-CD/tk-rep	Bunky bez funkčného p53	E1B-55kD delécia
	adMycTK	myc exprimujúce bunky	Myc-Max viažuci motív
Herpes simplex	G207	Proliferujúce bunky	Disrupcia ribonucleotid reduktázy a delécia gamma 34.5
	NV1020	Proliferujúce bunky	Delécia gamma 34.5
	1716	Proliferujúce bunky	Delécia gamma 34.5
Vakcínia	wild type +/- GM-CSF	neznáma	wild type
Vírus newcastelskej choroby	73-T, PV 701, kmeň Ulster, MTH-68/N	neznáma	wild type
Autonómne parvovírusy	H-1	Transformované bunky-↑proliferácia, ↓difefenciácia, ras, p53 mutácia	wild type
Reovírus	Reolysin	Aktivácia ras-signálnej dráhy	wild type

Tabuľka 1. Prehľad onkolytických vírusov v klinických testoch (modifikované podľa Kirn DH Replication-selective microbiological agents: fighting cancer with targeted germ warfare. *J Clin Invest* 2000;105:837- 839)

2.1.6 ONKOLYTICKÁ VÍRUSOVÁ TERAPIA

2.1.6.1 Onkolytiký herpes simplex vírus-1

Replikačne-kompetentné onkolytické HSV-1 použité v klinických protokoloch pre gliómy zahŕňajú G207 a HSV1716. Dvojitý mutant G207 herpes simplex vírus nesie delécie obidvoch kópií neurovirulentného génu γ34.5 a obsahuje inzerčnú inaktiváciu v U_L39 lokuse, ktorý kóduje ICP6, podjednotku vírusovej ribonukloetidreduktázy esenciálnej pre vírusovú replikáciu

v postmitotických bunkách (Markert J.M. et al., 2000). HSV1716 vírus je podobný G207, tiež mu chýbajú obidve kópie γ34.5, ale nesie nezmutovaný ICP6 gén (Rampling R. et al., 2000). Popri jeho neodmysliteľnej úlohe v patogenite HSV-1 v neurónoch, proteínový produkt γ34.5 ICP34.5 je zahrnutý v premáhaní obrany hostiteľskej bunky proti infekcii mediovanej proteín kinázou R (PKR). Pri infekcii, aktivácia PKR zastaví transláciu v infikovanej bunke fosforyláciou a inaktiváciou eukaryotického iniciačného faktoru 2 (eIF-2α). ICP34.5 naviaže a použije proteínovú fosfatázu 1-α na defosforyláciu eIF-2α, takže proteosyntéza môže pokračovať. Vírusy bez γ34.5 sa rozmnožujú v bunkách s defektnou PKR cestou, ako sú napríklad nádorové bunky so zvýšenou expresiou *ras*, zatiaľčo sa nemôžu efektívne rozmnožovať v bunkách s funkčnou PKR cestou (Farassati F. et al., 2001). Navyše, delécie v γ34.5 géne takisto odstraňujú sekvencie kódujúce latentne aktivované transkipty a takto znemožňujú týmto vírusom dostať sa do latentného stavu v rámci hostiteľskej bunky (Whitley R.J. et al., 1993). Inaktivácia ICP6 dodáva vírusu ďalšie bezpečnostné vlastnosti, ako napríklad schopnosť replikácie jedine v deliacich sa bunkách (Goldstein D.J. a Weller S.K., 1988) a zvýšenú senzitivitu k acykloviru a gancykloviru (GCV) (Mineta T. et al., 1994).

Otitrovanie dávky vírusu G207 bolo skúmané v klinickej štúdii fázy 1 s pacientami s recidivujúcimi malígnymi gliómami (Markert J.M. et al., 2000). Všetci pacienti boli liečení stereotaktickou intratumorálnou injekciou vírusu v dávke až do $3x10^9$ PFU. U žiadneho z pacientov sa nevyvinula herpetická encefalitída ani sa neprejavila žiadna iná signifikantná toxicita, okrem mierneho neurologického zhoršnia, takže nemohla byť stanovená maximálna tolerovateľná dávka. Navyše, MRI robená mesiac po liečbe ukázala u 8 z 20 pecientov zmenšenie objemu nádorov oproti stavu pred inokuláciou. U jedného z týchto pacientov došlo k takmer úplnej regresii tumoru a ďalší traja vykázali relatívne dlhé časy prežitia (Markert J.M. et al., 2000).

Bezpečnosť a toxicita vírusu HSV1716 boli prvýkrát hodnotené v dávke nastavujúcej štúdii fázy 1 u deviatich pacientov s recidivujúcimi gliómami vo vysokom štádiu, z ktorých žiadny neprejavil známky encefalitídy ani po podaní najvyššej dávky 10^5 PFU (Rampling R. et al., 2000). Ako odpoveď na viroterapiu, päť pacientov bolo stbilizovaných, zatiaľ čo ďalší preogredovali. Účinnosť HSV1716 bolo hodnotená v nasledujúcej štúdii s 12 pacientami s high-grade gliómami, ktorým 4-9 dní po intratumorálnej injekcii vírusu, boli nádory chirurgicky odstránené na stanovenie vírusovej replikácie (Papanastassiou V. et al., 2002).

Infekčný vírus HSV1716 bol kultivovaný z exstirpovaných nádorov v dvoch prípadoch a PCR detegovaný vo ôsmych ďalších. HSV1716 vírusom inokulované nádorové tkanivo z jedného z pacientov bolo kultivované *in vitro* a testované na prítomnosť vírusu s negatívnymi výsledkami. Keď boli bunky re-infikované *in vitro*, u malej frakcie z nich nastala lýza a vírus sa šíril ďalej (Harland J. et al., 2002). HSV1716 bol taktiež použitý ako adjuvantné terapeutikum u pacientov s novo-diagnostikovanými alebo recidivujúcimi gliómami vysokého štádia, priamou injekciou do pooperačnej dutiny v mozgu, ktorá vznikla po odstránení nádoru (Harrow S. et al., 2004). Podľa klinického protokolu liečba pokračovala rádioterapiou alebo chemoterapiou. Je dôležité, že ani v tejto štúdii sa nevyskytla žiadna toxicita spôsobená HSV1716, čo potvrdzuje bezpečnosť onkolytického HSV-1. Nayyše, traja z dvanástich liečených pacientov vykázali dlhodobé prežitie bez prejavov ochorenia vrátane jedného, u ktorého došlo k zmenšeniu objemu nádoru (Harrow S. et al., 2004).

Ďalšia štúdia fázy I/II skúmala doporčenú dávku JS1/34.5-/47-/GM-CSF, onkolytického HSV-1 so zakódovaným ľudským faktorom stimulujúcim granulocytárne a makrofágové kolónie (GM-CSF), pre ďalšie štúdie v kombinácii s chemorádioterapiou u pacientov so spinocelulárnymi karcinómamimy hlavy a krku (SCCHN) (Harrington K.J. et al., 2010). Pacienti dostali chemorádioterapiu a zvyšujúce sa dávky JS1/34.5-/47-/GM-CSF vírusu intratumorálnou injekciou nasledovanou odstránením nádoru. Primárne výstupy mali byť bezpečnosť a doporučená dávka/dávkovanie pre ďalšie štúdie. Sekundárne zahŕňali protinádorovú aktivitu a takisto sa sledovalo prežitie a početnosť relapsov ochorenia. 17 pacientov bolo liečených bez omeškania chemorádioterapie alebo dávky limitujúcej toxicity. 14 pacientov (82.3%) preukázalo nádorovú odpoveď a kompletná patologická remisia nastala u 93% pacientov s disekciou na krku. HSV bol detegovaný v injikovaných i okolitých neinjikovaných nádoroch v dávkach vyšších ako pôvodná dávka čo značí úspešnú vírusovú repikáciu. Všetci pacienti boli séropozitívni na konci liečby. Žiadny z pacientov nevyvinul lokoregionálny návrat ochorenia a prežitie ochorenia bolo 82.4% s mediánom 29 mesiacov (Harrington K.J. et al., 2010).

2.1.6.2 Onkolytické adenovírusy

Adenovírusy nesúce mutácie vo včasných génoch E1A alebo E1B, ktoré sú zodpovedné za naviazanie a inaktivovanie niektorých proteínov zahrnutých v kontrole bunkového cyklu a apoptóze, ako napríklad členovia rodiny pRB a p53, sa prednostne replikujú a lyzujú nádorové

bunky. Na prípravu nádorovo-selektívnych onkolytických vírusov bol prebádaný a využitý obrovský rozsah konceptov (McCormick F., 2001, Nemunaitis J. a Edelman J., 2002). Vírusy pripravované vo firme ONYX boli navrhnuté tak, aby využitím funkcií chýbajúcich v nádorových bunkách dosiahli nádorovo-špecifické hostiteľské rozhranie. Zostrojený ONYX-015 je neschopný exprimovať E1B 55K, včasný adenovírusový proteín dôležitý pre degradáciu p53 a teda obídenie p53 mediované zastavenie bunkového cyklu a apoptózu (Biederer C. et al., 2002). O ONYX-015 sa preto myslelo, že sa selektívne replikuje v nádorových bunkách s chýbajúcim funkčným p53, čím sa stáva gén E1B 55K nepotrebný. Zatiaľčo sa ukázalo, že indukcia p53 v niektorých nádorových bunkách obmedzila ONYX-015 replikáciu, potvrdilo sa, že nádorová selektivita vyústila v novú funkciu nádorových buniek, a to schopnosť exportovať z jadra neskoré vírusové mRNA v neprítomnosti E1B 55K (O'Shea C.C. et al., 2004). Ďalším vírusom vyrobeným v ONYX chýba schopnosť blokovať RB funkciu a môžu sa teda replikovať jedine v bunkách bez funkčného RB génu (Heise C. a Kirn D.H., 2000, Johnson L. et al., 2002). Príbuzný vírus, delta-24 (s doplnkovou RGD povrchovou modifikáciou) čoskoro vstúpi do klinickej fázy skúmania liečby glioblastómov (Fueyo J. et al., 2003).

ONYX-015, jeden z prvých upravených adenovírusov, bol testovaný na viacerých ľudských nádoroch v klinických štúdiách fázy I/II, ktoré potvrdili jeho bezpečnosť a protinádorovú účinnosť, hlavne v kombinácii s chemo- a rádioterapiou (Barzon L. et al., 2004, Chiocca E.A., 2002). Titrovanie dávky vírusu ONYX-015 v klinickej štúdii fázy I sa previedlo na 24 pacientoch s recidivujúcimi malígnymi gliómami (Chiocca E.A. et al., 2004). Vírus bol injikovaný v dávke 10^7-10^{10} PFU do dutiny po resekcii nádoru. Žiadny z pacientov nevykazoval vážne nepriaznivé príhody v spojitosti s liečbou ONYX-015 a maximálna tolerovaná dávka vírusu nebola dosiahnutá. Nanešťastie bezpečnosť liečby nebola doprevádzaná podobnými pozitívnymi výsledkami čo sa týka účinnosti. U všetkých pacientov ochorenie progredovalo, s výnimkov jedného pacienta s anaplastickým astrocytómom, u ktorého prebiehalo ochorenie stabilizovane. Medián času nádorovej progresie bol 46 dní a medián prežitia 6,2 mesiaca. Zaujímavé bolo, že u dvoch pacientov, u ktorých prebehla druhá resekcia 3 mesiace po injekciii ONYX-015, bola dokázaná lymfocytická a plazmocytická infiltrácia v mieste vpichu (Chiocca E.A. et al., 2004).

Ďalší modifikovaný adenovírus Ad5.SSTR/TK.RGD s posilnenou infektivitou, exprimujúci terapeutický tymidín-kinázový samovražedný gén a somatostatínový receptor

(SSTR) bol testovaný v klinickej štúdii fázy I na pacientkách s recidivujúcimi gynekologickými nádormi (Zhang T. et al., 2011). Pacientky boli liečené trojdňovou intraperitoneálnou dávkou 1×10^9 až 1×10^{12} vírusu Ad5.SSTR/TK.RGD nasledovanou intravenóznym 14 dňovým podaním gancykloviru. Najčastejšie pozorované toxicity spôsobené vírusom boli bolestivé symptómy, ktoré sa prejavili u pacientiek liečených najvyššou dávkou. Maximálna toelrovaná dávka nebola stanovená. Päť pacientiek vykazovalo stabilizované ochorenie, u všetkých ostatných ochorenie progredovalo. Jedna pacientka so stabilizovaným ochorením vykázala kompletnú remisiu ochorenia a normalizáciu CA125 v ďalšom sledovaní. Štúdie vzoriek ascites zozbieraných v rôznych časových intervaloch pacientiek liečených vyššou dávkou vírsu, preukázali prítomnosť Ad5.SSTR/TK.RGD a HSV1-tk expresiu (Zhang T. et al., 2011).

2.1.6.3 Onkolytický vírus newcastelskej choroby

Vírus newcastelskej choroby, NDV, je vtáčí paramyxovírus nepatogénny pre ľudí v jedinej výnimke, a to raritnej prechodnej respiračnej infekcii vyskytujúcej sa u chovateľov hydiny. Niektoré atenuované kmene NDV prejavili onkolytické vlastnosti proti ľudským nádorom, zatiaľ čo nie neoplastické bunky ostali nedotknuté (Reichard K.W. et al., 1992). Mechanizmus účinku atenuovaného NDV ostáva stále neznámy, aj keď sa zdá, že aktivácia *Ras* signálnej dráhy bude hypoteticky zohrávať rolu ako sa predpkladá i u γ34.5-defektného HSV-1. NDV taktiež prejavuje mnohopočetné imuno-modulačné vlastnosti (Schirrmacher V. et al., 1999) a indukuje produkciu dvojvláknovej RNA, interferónov a chemokínov v infikovaných bunkách (Washburn B. a Schirrmacher V., 2002).

Onkolytický NDV PV701 bol použitý v klinickej štúdii fázy I zahrňujúcej 79 pacientov s rôznymi pokročilými solídnymi nádormi (Pecora A.L. et al., 2002). Výsledky tejto štúdie potvrdili, že optimálna liečba bez nežiaducich účinkov má začať nízkymi dávkami s postupným zvyšovaním dávok. Najčastejšie negatívne účinky boli zvýšená teplota a symptómy podobné chrípke, avšak vyskytli sa aj závažné komplikácie, vrátane smrti spôsobenej liečbou. Ďalší onkolytický NDV , MTH-68/H, bol skúmaný v štúdiách fázy I/II u pacientov s pokročilými nádorovými ochoreniami (Csatary L.K. et al., 1993, Csatary L.K. et al., 1999) a u 14 pacientov s gliómami vysokého štádia (Csatary L.K. et al., 2004).

Liečba pozostávala z dennej intravenóznej injekcie $2x10^7$ PFU MTH-68/H vírusu. Dávky boli postupne dvíhané až na $2x10^8$ PFU denne a v prípade klinického zlepšenia udržiavané počas celého života pacienta v dlhších intervaloch. Žiadne negatívne pozorovania počas trvania štúdie neboli zaznamenané. Zo 14 pacientov, 4 pacienti s glioblastoma multiforme mali čas prežitia medzi 5 a 9 rokov vrátane dvoch pacientov, ktorí preukázali takmer úplné zmnešenie nádoru (Csatary L.K. et al., 2004).

Najnovšia štúdia fázy I/II s NDV-HUJ onkolytikým vírusom u pacientov s pokročilým recidivujúcim glioblastoma multiforme priniesla priaznivé výsledky. (Freeman A.I. et al., 2006) Postupne stupňovaná dávka vírusu bola pacientom podávaná v intravenóznej infúzii po dobu 15 minút v rôzne početných cykloch. Všetci pacienti bez progresie ochorenia boli udržiavaní dvomi dávkami po 11 biliónov jednotiek vírusu i.v. týždenne. Toxicita bola minimálna s výskytom horúčky stupňa I/II u piatich pacientov. Maximálna tolerovaná dávka nebola dosiahnutá. Anti-NDV hemaglutinín protilátky boli preukázané za 5-29 dní. NDV-NUJ bol detegovaný z krvi, slín, vzoriek moču a jednej tumor biopsie. Jeden pacient dosiahol komletnej odpovede (Freeman A.I. et al., 2006). Zistenie dobrej tolerancie a nádejné výsledky oprávňujú k ďalšiemu testovaniu NDV-HUJ na glioblastómoch ako i na iných druhoch nádorového ochorenia. Tieto výsledky klinických štúdií vyzerajú veľmi priaznivo, ale kontrolované štúdie sú potrebné na konečné zadefinovanie bezpečnosti a onkolytickej aktivity NDV.

2.1.7 KLINICKÉ TESTOVANIE ONKOLYTICKÝCH VÍRUSOV

Z výsledkov preklinických modelov je evidentné, že onkolytické vírusy majú obrovský potenciál stať sa novým dôležitým protinádorovým terapeutikom. Výsledky fáz I a II klinických testov intratumorálne i systémovo podávaných vírusov sa pomaly začínajú sumarizovať. Zdá sa, že súčasne používané onkolytické vírusy sú bezpečné a v porovnaní s mnohými konvenčnými cytostatikami majú menej závažné akútne nežiaduce účinky (Markert J.M. et al., 2000, Mulvihill S. et al., 2001). Prvý klinicky testovaný onkolytický vírus je adenovírus ONYX-015, ktorý bol od roku 1996 predmetom skúmania 18 klinických štúdií fázy I a II. Do dnešných dní bolo viac než 250 pacientov liečených ONYX-015 a viac než 170 vírusom newcastelskej choroby (Chernajovsky Y. et al., 2006). Jasné dôkazy z preklinických i klinických štúdií nastoľujú predpoklad, že kombinácia replikačne-

kompetentných vírusov so štandardnou protirakovinovou liečbou ako je operačná liečba, chemoterapia a rádioterapia môže vyústiť v obrovský terapeutický prínos (Freytag S.O. et al., 1998, Heise C. et al., 1997, Khuri F.R. et al., 2000, Rogulski K.R. et al., 2000, Zhang Q. et al., 2004). ONYX-015 sa stal prvým vírusom, ktorý vstúpil do klinického testovania v kombinácii s chemoterapiou (Aghi M. a Martuza R.L., 2005). V niektorých prípadoch terapia vírusom v kombinácii s chemoterapiou bola natoľko úspešná, že bolo zahájené klinické testovanie fázy III (Aghi M. a Martuza R.L., 2005, Lamont J.P. et al., 2000, Nemunaitis J. et al., 2000, Nemunaitis J. et al., 2001).

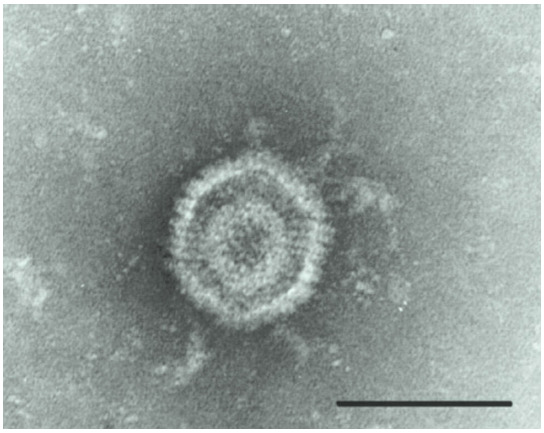
Väčšina dodnes uskutočnených klinických štúdií zahŕňala intratumorálne podávanie. Systematická liečba rakoviny onkolytickými vírusmi je jasným ďalším krokom vedúcim k ich širšiemu uplatneniu. Nedávna intravenózna administrácia PV701 a ONYX-015 pacientom v pokročilom štádiu spinocelulárnych karcinómov hlavy a krku a karcinómov gastrointestinálneho traktu s metastázami do pečene zahŕňala štúdium maximálnej tolerovanej dávky (Nemunaitis J. et al., 2001, Reid T. et al., 2002). Adenovírus ONYX-015 bol podávaný pacientom s gastrointestinálnym karcinómom diseminovaným do pečene infúzne cez hepatickú artériu (Pecora A.L. et al., 2002). Napriek tomu je potrebné zahájiť a vylepšiť viac klinických štúdií a preskúmať oboje, loko-regionálne i systematické podávanie. Potrebujeme zistiť, ako dôveryhodne preklinické modely určujú výsledok testovania na ľudských jedincoch. Je celkom pravdepodobné, že na otázky týkajúce sa virémie, odbúravania vírusu, humorálnej a celulárnej imunitnej odpovede, schopnosti šírenia z nádoru na nádor a stability vírusu v ľudskom organizme odpovie len testovanie na ľuďoch. Jemné dolaďovanie a optimalizácia vírusových terapeutík sa najlepšie preveria v klinických štúdiách fázy II a III.

2.2 REOVÍRUS

2.2.1 VÝSKYT A TAXONOMICKÉ ZARADENIE REOVÍRUSU

Reovírusy, prototypní členovia rodiny *Reoviridae*, boli prvýkrát izolované z tráviaceho traktu jedincov, ktorí nejavili známky ochorenia. Tieto vírusy vykazujú vrodenú preferenciu replikácie v mnohých transformovaných bunkách.

Reovírus (akronymum pre anglické *r*espiratory *e*nteric *o*rphan virus – respiračnoenterický vírus sirota) je vírus s vysokou prevalenciou v ľudskej populácii, avšak nie je asociovaný so žiadnym známym ochorením (Tyler K.L., 2001). Bol izolovaný z dýchacieho a tráviaceho traktu zdravých jedincov a pokladá sa za vírus - sirotu, pretože infekcii, ktorú spôsobuje, chýbajú klinické príznaky (SABIN A.B., 1959). V prírode sa vyskytuje v odpadových a stojatých vodách. Už polovica detí vo veku 12 rokov nesie proti nemu protilátky a väčšina dospelých jedincov bola počas svojho života exponovaná. Ako spomenuté vyššie, reovírus je nepatogénny, čo znamená, že pozitívnym jedincom chýbaju známky infekcie. Spojitosť reovírusu so schopnosťou zabíjať rakovinové bunky bola objavená keď sa ukázalo, že reovírus je schopný reprodukcie v rôznych nádorových bunkových líniách. Serotyp 3 Dearing sa testuje v jeho pôvodnej, prirodzenej a nezmutovanej forme. Na obrázku č.1 je elektrónoptický obraz reovírusuu. Taxonomicky patrí do rodiny *Reoviridae*. Je to rodina neobalených vírusov s takmer guľatým, ikosahedrálnym tvarom, veľkosťou v rozmedzí od 70 do 85nm. Skladajú sa z vnútorného jadra (veľkosť 60-70nm) a vonkajšej proteínovej kapsidy. Ich genóm je segmentovaný a pozostáva z 10-12 dvojvláknových RNA o celkovej veľkosti 24 kb. Reovírus je zástupcom druhu *Reoviridae*, ktorý infikuje ľudí (Tyler K.L., 2001).



Obrázok č. 1 Reovírus, elektrónový mikrokop.

2.2.2 SELEKTÍVNA REPLIKÁCIA REOVÍRUSU A RAKOVINA

Rozdiely medzi vírusovo alebo spontánne transformovanými bunkovými líniami a primárnymi alebo netransformovanými bunkami s prihliadnutím na ich citlivosť voči reovírusovej cytotoxicite boli prvýkrát zaznamenané v roku 1977 (Hashiro G. et al., 1977). Podobne, ľudské pľúcne fibroblasty (WI-38) vykazovali silnejšiu poddajnosť reovírusovej replikácii po trnasformácii 40 T antigénom simianského vírusu (Duncan M.R. et al., 1978). Avšak až v deväťdesiatych rokoch minulého storočia boli odhalené niektoré z kľúčových faktorov molekulárnych základov selektívnej replikácie reovírusu v transformovaných bunkách.

Receptorová špecificita často diktuje tropizmus vírusov (Cohen J.A. et al., 1988, Schneider-Schaulies J., 2000, Sieczkarski S.B. a Whittaker G.R., 2005). Keď sa začal výskum činiteľov určujúcich selektívnu onkolýzy reovírusu, bolo známe, že vláknitý chvost reovirálneho pripájacieho proteínu, σ1, sa viazal na sialovú kyselinu (Armstrong G.D. et al., 1984, Dermody T.S. et al., 1990). Ubikvitárna povaha sialovej kyseliny však poukazovala na neveľké rozdiely v bunkovej odolnosti voči reovírusu. Keďže aberácie týkajúce sa tyrozínkinázových receptorov sú často spájané s transformáciou, použitie týchto receptorov pre reovírusový prienik predkladalo potenciálne prepojenie medzi transformáciou a prienikom reovírusovej infekcie. EGFR-defektné myšacie bunkové línie (NR6 a B82) boli relatívne rezistentné voči reovírusu, kdežto transfekcia EGFR do týchto bunkových línií dokázala signifikantne zvýšiť reovírusovú infekciu (Strong J.E. et al., 1993). Zistilo sa, čo je zaujímavé, že zahrnutie EGFR v reovirálnej replikácii je závislé na signálnych dráhach iniciovaných cez tyrozínkinázový receptor skôr než na receptor viažucej špecificite (Shmulevitz M. et al., 2005). Mutovaný EGFR zbavený signálnych schopností bol neschopný umožniť reovirálnu infekciu, zatiaľ čo v-erbB onkoproteín, ktorému chýba extracelulárny ligand-viažuca doména ale obsahuje konštitutívne aktívnu kinázovú doménu, bol schopný umožniť postačujúcu reovírusovú infekciu (Aghi M. a Martuza R.L., 2005, Strong J.E. et al., 1993, Strong J.E. a Lee P.W., 1996). Preto to, čo začalo ako hľadanie sekundárneho receptora pre reovirálny vstup, ktorý by napomohol selektívnej replikácii v transformovaných bunkách, sa zmenilo na veľmi zaujímavú spojitosť medzi statusom intracelulárnej signálnej dráhy a reovirálnym lytickým cyklom.

2.2.2.1 Ras/RalGEF/p38 signálna dráha v replikácii reovírusu

Interakcia rastových faktorov s im príbuznými receptormi vyúsťuje v spustení kaskády intracelulárnych biochemických udalostí, čo vedie k aktivácii viacerých bunkových funkcií. Ktoré bunokvé funkcie môžu ovlyvniť replikáciu reovírusu? A ktoré komponenty signálnych dráh sú dôležité? EGFR signálna kaskáda začína oligomerizáciou receptora a následnou aktiváciou tyrozínkinázovej aktivity, čo vyústi v autofosforyláciu špecifických tyrozínových reziduí (Fantl W.J. et al., 1993, Ullrich A. a Schlessinger J., 1990, Weiss A. a Schlessinger J., 1998), Adaptorové molekuly s fosfotyrozínovým viazaním, src homológne domény 2 (SH2), sú pribrané na aktiváciu EGFR a následne so sebou strhnú skupinu proteínov špecifických pre jednu z mnohých pokračujúcich kaskád (Buday L., 1999, Pawson T., 1994, Pawson T. a Scott J.D., 1997). *Ras* signálna dráha je hlavná dráha nasledujúca po EGFR (Campbell S.L. et al., 1998). *Ras* proteíny tvoria podrodinu malých GTP-viažucich proteínov zahrnutých v regulácii širokého spektra bunkových funkcií ako je bunkový rast, diferenciácia a bunkové prežitie. Membránové *Ras* proteíny oscilujú medzi inaktívnym GDP-viažucim stavom a aktívnym GTP-viažucim stavom. Aktivácia *Ras* proteínov je umožňovaná guanínnukleotid výmennými faktormi ako je Son of Sevenless (Sos), ktoré sú priberané vyššie popísanými adaptorovými molekulami na aktiváciu EGFR (Overbeck A.F. et al., 1995). S takto dôležitou funkciou v bunkovej odpovedi na aktiváciu EGFR, sa *Ras* signálna dráha stáva logickým cieľom pre možný súvis v pôsobení reovírusu.

Dokonalá analýza ukázala, že aktivovaná *Ras* signálna dráha hrá dôležitú rolu v infekcii reovírusu. Bolo zistené, že bunkové línie konštitutívne exprimujúce aktívne onkogény Sos alebo *Ras* podliehajú reovírusovej infekcii (Strong J.E. et al., 1998). Dôležité bolo zistenie, že keď je aktivovaný *Ras* proteín umiestnený za zinkom-indukovaný promoter, produktívna infekcia reovírusom sa vyskytuje jedine v prítomnosti $ZnSO_4$.

Z viac než 18 nižšie postavených efektorov Ras sú najlepšie charakterizované Raf kinázy, fosfatidylinozitol 3 kináza (PI3-kináza) a guanín výmenné faktory (GEFs) z malej G proteín Ral dráhy. Opisy týchto dráh sú za hranicami rozsahu tejto práce, ale boli popísané v mnohých publikáciách (Campbell P.M. a Der C.J., 2004, Repasky G.A. et al., 2004, Reuther G.W. a Der C.J., 2000). Ďalšie laboratóriá skúmajúce implikácie *Ras* aktivácie vytvorili mnohé užitočné nástroje na rozlišovanie medzi po sebe nasledujúcimi kaskádami (Khosravi-Far R. et al., 1996, Rodriguez-Viciana P. et

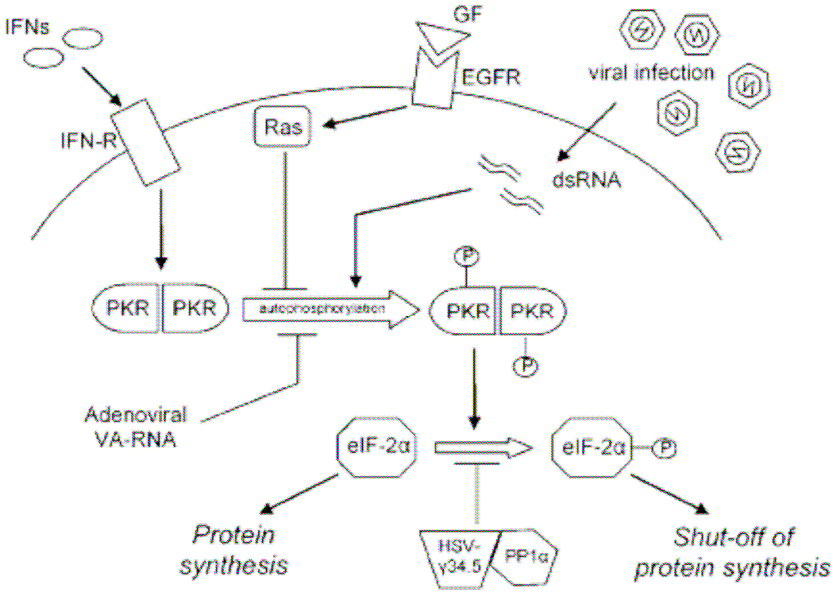
al., 1997, White M.A. et al., 1995). Mutanty s konštitutívnou *Ras* aktiváciou (RasV12) s aditívnymi mutáciami brániacimi v naviazaní na špecifické efektorové molekuly PI3K, RalGEF alebo Raf ukázali, že reovírusová infekcia je nezávislá na signalizácii cez Raf alebo PI3-kinázové kaskády (Norman K.L. et al., 2004). Navyše aktívny mutant RalGEF povolil účinné rozmnožovanie reovírusu i v neprítomnosti aktivovaného *Ras*. Dominnantne-negatívny mutant Ral, cieľový proteín RalGEF-mediovanej GDP/GTP výmeny, spôsobil, že *Ras*-transformované bunky boli nepriepustné pre reovírus. Z týchto signifikantných dát teda vyplýva, že Ras/Ral/GEF dráha hrá rolu v selektívnej replikácii reovírusu (Norman K.L. et al., 2004).

Signálne dráhy poniže *Ras* sú enormne komplikované vysokým stupňom prepojenia a závislosti na faktoroch ako je bunkový pôvod či extracelulárne prostredie. Keďže je replikácia reovírusu závislá na jednej alebo viacerých častiach *Ras* signálnej dráhy, toto zistenie poskytuje značný priestor na hľadanie komponentov nasledujúcich za *Ras*. Predošlé pozorovania, že reovirálna reprodukcia je posilňovaná v bunkách vystavených stresovým podmienkam ako UV, urýchlili analýzu úlohy stresom-aktivovaných proteínkináz. Štúdie ukázali, že p38, ale nie c-Jun-N-terminálna kináza (JNK) participuje v nastolení infektivity reovírusom. Inhibítor aktivácie p38 (SB203580) potlačil reovirálnu replikáciu v bunkách transfekovaných aktivovaným *Ras* a RalGEF (Norman K.L. et al., 2004). Dáta získané s inhibítorom p38 naznačujú, že p38 je efektorom nasledujúcim za RalGEF, tento vzťah bol popísaný už Ouwensom (Ouwens D.M. et al., 2002). Alternatívne, RalGEF a p38 môžu takisto slúžiť ako komponenty rozdielnych dráh spojených pre spoločný účel. Chýbajúce zložky medzi RalGEF aktiváciou, p38 aktivitou a reovirálnou replikáciou ostávajú byť ešte objavené. Doposiaľ nie je jasné, ktoré efektory slúžia na mediáciu RalGEF-p38 konexie a ktoré kaskády ležia pod RalGEF a p38.

2.2.2.2 Reovírus a *Ras* signálna dráha

Ako spomínam vyššie, reovírus sa prednostne množí v nádorových bunkách, ktoré majú aktivované gény rodiny *Ras*, alebo kompletnú *Ras* - signálnu dráhu (Norman K.L. a Lee P.W., 2000). Aktivovaný *Ras* (alebo jeho signálna kaskáda) je prítomný u 60-80% ľudských malignít (Coffey M.C. et al., 1998). Štúdie ukázali, že reovírus nie je schopný produktívne infikovať myšacie embryonálne fibroblasty NIH-3T3, pokiaľ neexprimujú aktívny *Ras* (Strong J.E. et al., 1998). Dôvod, prečo bunky s aktívnou *Ras* signálnou

dráhou môžu byť úspešne infikované reovírusom, úzko súvisí s inhibíciou bunkovej ochrany proti vírusovej infekcii. Po primárnej transkripcii v netransformovaných, reovírusom infikovaných bunkách je aktivovaná PKR (dvojvláknovou RNA aktivovaná proteínkináza). Fosforyláciou alfa podjednotky iniciačného faktora 2, eIF2-α, PKR zastaví proteosyntézu. Táto fosforylácia je však inhibovaná ak je *Ras* cesta aktívna, povoliac transláciu vírusových mRNA a následný vstup vírusu do vírusového lytického cyklu. Schéma vplyvu aktivácie *Ras* na replikáciu reovírusu je znázornená na obrázku č.2 V nádorových bunkách s aktívnou *Ras* signálnou dráhou sa reovírus môže voľne replikovať, čím zabíja hostiteľské nádorové bunky (Strong J.E. et al., 1998). Následkom bunkovej smrti novovzniknuté vírusové častice voľne infikujú susediace nádorové bunky. Tento cyklus infekcie, replikácie a bunkovej smrti sa za ideálnych podmienok môže opakovať až pokiaľ neostanú prítomné žiadne nádorové bunky nesúce *Ras* aktiváciu.



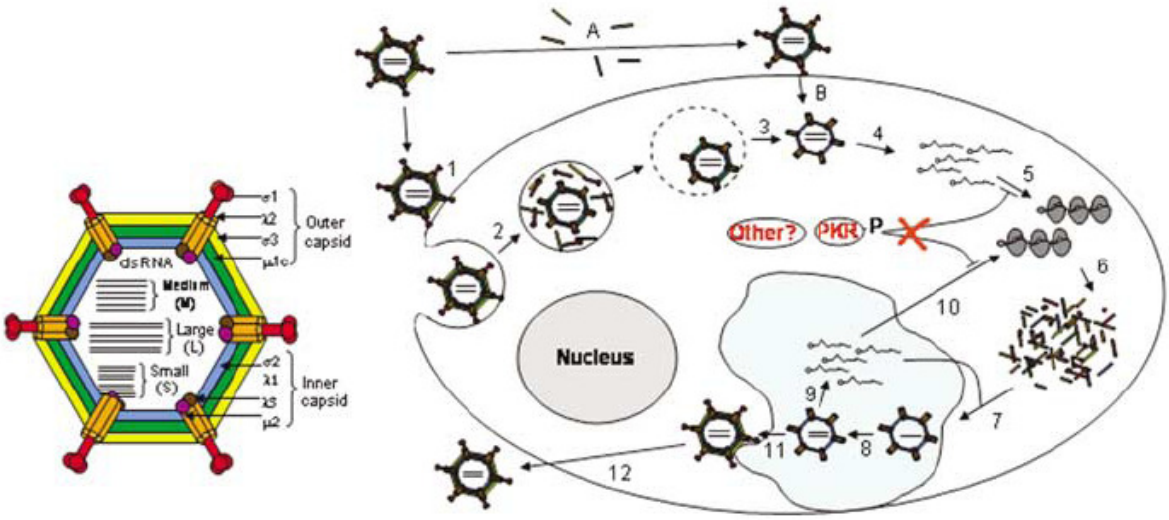
Obrázok č. 2 Vplyv aktívneho Ras proteínu a aktivácie PKR na replikáciu reovírusu.

2.2.2.3 Replikačný cyklus reovírusu

Lytický cyklus reovírusu začína jeho naviazaním na zvyšok kyseliny sialovej v povrchovom receptore hostiteľa. Naviaže sa naň cez trimérický σ1 napájací proteín (attachment protein), ktorý prečnieva z 12 vrcholov ikosahedrálnej kapsidy (Lee P.W. et al., 1981). Po naviazaní sa vytvoria klatrínom potiahnuté jamky (clathrin-coated pits) a vírus vstúpi do bunky receptorom sprostredkovanou endocytózou. Vo vzniknutom endozóme/lyzozóme začína na kyslom prostredí závislá proteolýza kapsidových proteínov σ3 a μ1/μ1c, čím vzniká intermediárna subvirálna častica (ISVP). Neskôr sa degraduje σ3 proteín, čo teoreticky sprístupní proteín μ1/μ1c, ktorý umožní penetráciu ISVP cez lyzozomálnu membránu. Bolo dokázané, že μ1/μ1c je schopný narušovať membránové dvojvrstvy *in vitro* (Lucia-Jandris P. et al., 1993). μ1/μ1c je myristoylovaný, čo taktiež napomáha fúzii ISVP s membránou (Nibert M.L. et al., 1991).

Po tomto kroku nasleduje primárna transkripcia 10 čiapočkovaných transkriptov po celej ich dĺžke, mediovaná virálnou na dvoj-vláknovej RNA- závislou RNA polymerázou (dsRNA dependent RNA polymerase). Primárne transkripty sú prekladané pomocou hostiteľského replikačného aparátu. Následne asociujú s produktami primárnej translácie a vytvárajú RNA zmesné komplexy (RNA assortment complexes). Finálna syntéza mínusového vlákna genómovej RNA sa odohráva v týchto vznikajúcich komplexoch. Po nej môže začať sekundárna transkripcia neskorých vírusových mRNA. Syntéza vírusovej mRNA v rámci vírusovej sub-častice je charakteristickým znakom reovírusovej replikácie. Syntéza neskorých vírusových proteínov zo sekundárnych transkriptov často koreluje so zníženou syntézou proteínov hostiteľskej bunky (Zweerink H.J. a Joklik W.K., 1970). Záverečné poskladanie proteínovej kapsidy dáva vznik novým reovírusovým časticiam, čo vedie k následnej lýze hostiteľskej bunky a jej smrti. Schematicky na obrázku č.3.

V infikovanej bunkovej kultúre je maximálny vírusový výťažok dosiahnutý 15-18 hodín post-infekčne, s 200-2000 plaky tvoriacich jednotiek na bunku (PFU, plaque forming units). Pomerne typickým znakom pre kultivovanú populáciu vírusu je vysoký pomer infekčných a existujúcich častíc (1: 100 až 1:1000). Toto sa deje pravdepodobne kvôli predominantnej prítomnosti nezrelých, defektných častíc vznikajúcich počas vírusovej replikácie. Reovírusy sú pomerne dlho stabilné a sú rezistentné voči expozícii vysokým iónovým silám, relatívne vysokým teplotám (prekračujúcich 50˚C) a extrémnym zmenám v pH.



Obrázok č. 3. Replikácia reovírusu.

Štádiá životného cyklu reovírusu:

(1) naviazanie na bunku pomocou interakcie σ1so zvyškom kyseliny sialovej a/alebo JAM receptorov na cieľových bunkách
(2) receptorom sprostredkovaná endocytóza indukuje uvoľnenie σ1 a σ3
(3) endocytickými proteázami odštiepený μ1c rozvolní endozóm
(4) objavuje sa primárna transkripcia vnútri reovírusových jadier a čiapočkované mRNA sú uvoľnené
(5) primárna translácia všetkých mRNA pomocou hosťovského aparátu
(6) akumulácia virálnych proteínov vo „vírusových továrňach“ tvorených vírusovými neštrukturálnymi proteínmi
(7) vytvorenie nových jadier
(8) syntéza mínusovej RNA
(9) sekundárna transcripcia vnútri nových vírusvových jadier
(10) množenie vírusových proteínov pomocou sekundárnej translácie
(11) kompletné zostavenie vonkajšej kapsidy
(12) uvoľnenie nasledované lýzou bunky
(A) intestinálne proteázy produkujú intermediárne subvirálne častice (ISVPs);
(B) ISVPs prenikajú priamo cez membránu bunky. PKR a zrejme ďalšie neidentifikované translačné kontrolné elementy blokujú primárnu a/alebo sekundárnu reovirálnu preteínovú transláciu.*Ras* aktivácia uvoľňuje translačný blok v transformovaných bunkách.

2.2.2.4 Reovirálna translácia je blokovaná v netransformovaných bunkách

Selektívna replikácia reovírusu v *Ras*-transformovaných bunkách je evidentná po preskúmaní stupňa expresie reovírusových proteínov a formácie novovzniknutých reovirálnych partikulí. Čo ale ostáva byť odhalené je presný mechanizmus, ktorým *Ras* signálna dráha ovplyvňuje reovirálny životný cyklus.

Reovírus prekonáva rovnaké naviazanie, vstup a primárnu transkripciu v oboch, transformovaných i normálnych bunkách (Norman K.L. et al., 2004, Strong J.E. et al., 1998). Naopak, proteínová expresia monitorovaná ^{35}S-metionínovým značením a imunofluorescenčnou mikroskopickou detekciou so špecifickými reovírusovými protilátkami ukázala, že expresia reovirálnych proteínov je vysoko nedostačujúca v netransformovaných bunkách. Zvýšená expresia reovirálnych proteínov v *Ras*-transformovaných bunkách koreluje so zvýšeným virálnym titrom. Súčasné analýzy teda naznačujú, že blok v reovirálnej replikácii leží medzi primárnou transkripciou a expresiou proteínov. Z virologickej i nádorovej perspektívy bude zaujímavé poznať presné procesy blokovania reovirálnej replikácie v neprítomnosti *Ras* aktivácie.

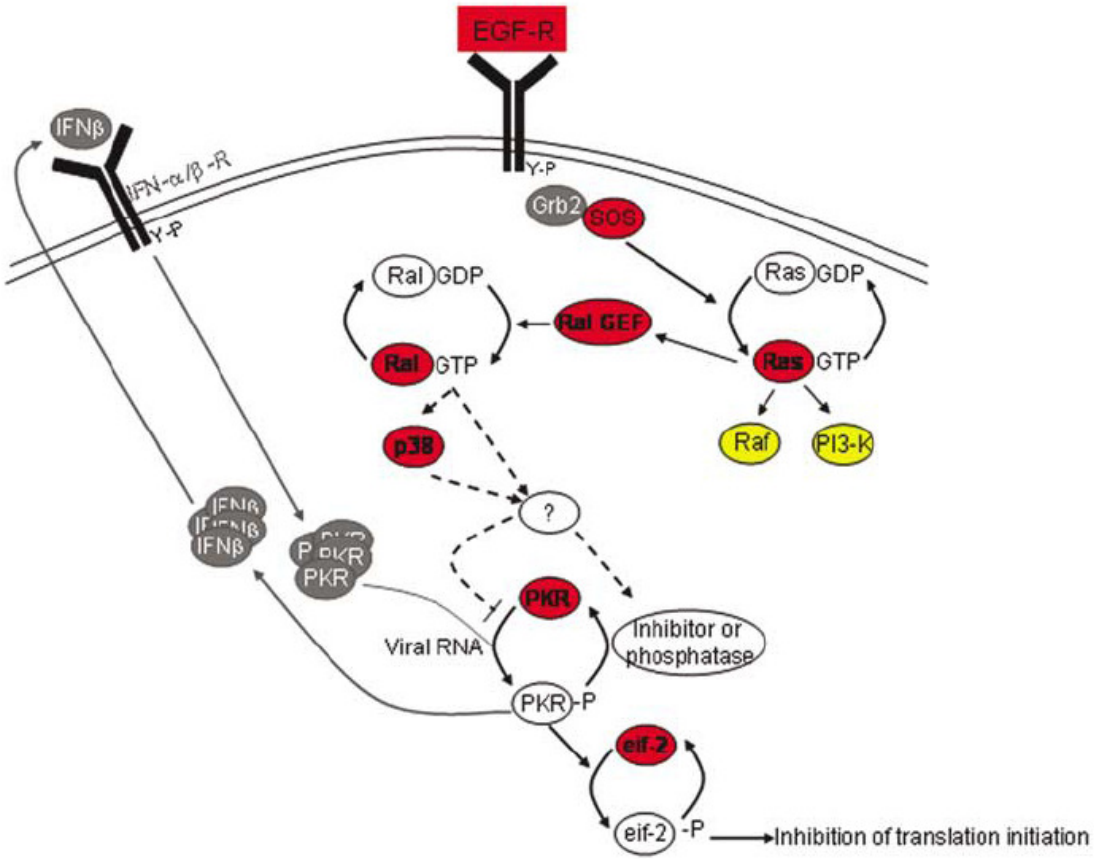
2.2.2.5 Vplyv aktivácie PKR na reovirálnu transláciu

Hlavný mechamizmus zastavenia iniciácie translácie v odpovedi na vírusové infekcie a environmentálny stres zahŕňa fosforyláciu alfa podjednotky eukaryotického iniciačného faktoru-2 (eIF2-α) na na dvojvláknovej-RNA-závislej proteín kinzáze (PKR) (Brostrom C.O. a Brostrom M.A., 1998, de H.C. et al., 1996, Wek R.C., 1994). Napriek tomu, že v bunke je vždy prítomná nízka hladina PKR, jej expresia sa zvyšuje ako odpoveď na IFN uvoľnený vírusmi infikovanými bunkami. Naviazanie na dvojvláknovú RNA vyústi v dimerizáciu PKR, jej autofosforyláciu a aktiváciu. Fosforylácia eIF2-α aktivovanou PKR zabráni formácii GTP-naviazaného eIF2-α a tým predíde pripojeniu Met-tRNA a formácii 43Siniciačného komplexu (Mohr I., 2004).

Možná účasť PKR v reovirálnej replikácii bola naznačená dvomi pozorovaniami: S1 mRNA segment reovírusu sa ukázal byť potentným aktivátorom PKR (Bischoff J.R. a Samuel C.E., 1989) a translácia reovirálnej mRNA bola v netransformovaných bunkách narušená oproti bunkám s aktivovaným *Ras*. Použitím štandardnej *in vitro* kinázovej eseje na

detekciu aktivovanej PKR Strong et al. (Strong J.E. et al., 1998) zistili, že PKR bola fosforylovaná v netransformovaných NIH 3T3 bunkách v odpovedi na replikáciu reovírusu. Nedostatok fosforylácie PKR v reovírusom infikovaných *Ras*-transformovaných bunkách naznačuje, že reovirálna translácia chýba v transformovaných bunkách, pretože PKR nie je aktivovaná. Tieto štúdie nestanovujú, či aktivácia PKR je príčina alebo dôsledok bunkových zmien prospešných pre infekciu reovírusom. Relatívne špecifický inhibítor fosforylácie PKR obnovil transláciu reovírusu v netransformovaných bunkách, čím podal dôkaz priamej úlohy PKR v nastoľovaní rezistencie reovirálnej replikácie. Expresia reovirálnych proteínov bola nedostatočná v normálnych ($PKR^{+/+}$) myších embryonálnych fibroblastoch (MEFs) ale v $PKR^{-/-}$ MEFs bola postačujúca. Spojitosť medzi PKR inaktiváciou a *Ras* signalizáciou už bola spomenutá a môže byť potvrdená zisteniami o selektivite reovirálnej replikácie (Mundschau L.J. a Faller D.V., 1994). V súčasnosti sa pozorovania prikláňajú k názoru, že aktivácia PKR hrá rolu v určovaní rezistencie voči infekcii reovírusom.

Mnohé otázky týkajúce sa vzťahu PKR a replikácie reovírusu ostávajú na zodpovedanie. Aký je mechanizmus zvýšenej aktivity PKR v normálnych bunkách: zvýšená expresia PKR, zvýšená PKR fosforylácia, a/alebo znížená aktivita fosfatáz, ktoré pôsobia na PKR a jej substráty? Akú úlohu, ak vôbec, hrajú IFNs a aktivácia dsRNA v rozlišovaní výsledkov reovirálnej infekcie v transformovaných a nezmenených bunkách? Aké je molekulárne prepojenie medzi Ras/RAlGEF/p38 signálnou dráhou a PKR aktiváciou? Je PKR jediná podmienka úspešnej replikácie reovírusu? Aké ďalšie efektory môžu hrať rolu v upravovaní úspešnosti v translácii reovirálnej mRNA?



Obrázok č. 4. Signálne dráhy a efektory zahrnuté v priebehu reovirálnej infekcie. Proteíny, ktoré sa podieľajú na reovirálnej infektivite sú znázornené červenou, zatiaľ čo tie, ktoré nie sú nevyhnutné pre jeho replikáciu sú žlté. Zvýšená expresia IFNβ a PKR je v obrázku znázornená viacerými kópiami. Úloha IFN, IFN recepetoru a Grb2 (sivá) v reovirálnej onkolýze nebola testovaná. Čiarkovanou čiarou sú znázornené dráhy, ktoré sú prepojené doteraz neznámymi intermediárnymi komponentami.

2.2.3 KLINICKÉ VYUŽITIE REOVÍRUSU

V roku 1998 bola v Calgary, Alberta, Kanada, založená spoločnosť, ktorá sa zaoberá skúmaním onkolytických schopností reovírusu a jeho využitím a výrobou ako nového potenciálneho protinádorového terapeutika. Vyvinula technológie umožňujúce prípravu reovírusu sérotypu III kmeň Dearing schváleného na klinické použitie- Reolysin-u. Táto firma momentálne s Reolysin-om vedie dvanásť klinických štúdií fázy I a II v Spojenom Kráľovstve, Spojených Štátoch Amerických a Kanade a dvanásť ich úspešne ukončila. Prebiehajúci klinický program s Reolysin-om zahŕňa viaceré ľudské nádorové ochorenia a používa rôzne spôsoby podávania,

nevynímajúc lokálnu aplikáciu, systematickú administráciu a podávanie v kombinácii s rádioterapiou a chemoterapiou. V máji 2010 spoločnosť začala zápis do prvej klinickej štúdie fázy III, REO 018, skúmajúcej intravenóznu administráciu REOLYSIN®-u v kombinácii s paklitaxelom a karboplatinou u pacientov s na platinu odolnými nádormi hlavy a krku. Zhrnutie t.č. prebiehajúcich a ukončených klinických štúdií vedených firmou Oncolytics Biotech je v **Tabuľke č. 2.** (www.oncolyticsbiotech.com).

Číslo štúdie	Fáza	Názov štúdie	Miesto	Status
IND 213 (NCIC CTG Trial)	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel for Patients with Advanced or Metastatic Breast Cancer	Kanada	Ohlásená
IND 211 (NCIC CTG Trial)	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Docetaxel or Pemetrexed for Patients with Previously-Treated Advanced or Metastatic Non-Small Cell Lung Cancer	Kanada	Ohlásená
IND 210 (NCIC CTG Trial)	Fáza II	Intravenous Administration of REOLYSIN® in Combination with FOLFOX-6 Plus Bevacizumab (Avastin®) Versus FOLFOX-6 Plus Bevacizumab Alone in Patients with Advanced or Metastatic Colorectal Cancer	Kanada	Ohlásená
IND 209 (NCIC CTG Trial)	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Docetaxel for Patients with Recurrent or Metastatic Castration Resistant Prostate Cancer	Kanada	Ohlásená
OSU-11148 (NCI Trial)	Fáza I	Intravenous Administration of REOLYSIN® for Patients with Relapsed Multiple Myeloma	USA	Prebieha
OSU-10045 (NCI Trial)	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Metastatic Pancreatic Cancer	USA	Prebieha
COG-ADVL1014 (NCI / COG Trial)	Fáza I	Intravenous Administration of REOLYSIN® in Combination with Cyclophosphamide for Pediatric Patients with Relapsed or Refractory Solid Tumors	USA	Prebieha
GOG-0186H (NCI / GOG Trial)	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel for Patients with Persistent or Recurrent Ovarian, Fallopian Tube or Primary Peritoneal Cancer	USA	Prebieha
REO 022	Fáza I	Intravenous Administration of REOLYSIN® in Combination with FOLFIRI for Patients with Colorectal Cancer	USA	Prebieha

Číslo štúdie	Fáza	Názov štúdie	Miesto	Status
REO 021	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Squamous Cell Carcinoma Lung Cancer	USA	Prebieha
REO 020	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Metastatic Melanoma	USA	Prebieha
REO 018	Fáza III	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Platinum-Refractory Head and Neck Cancers	Medzi-národná	Prebieha
REO 017	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Gemcitabine for Patients with Advanced Pancreatic Cancer	USA	Prebieha
REO 016	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Non-Small Cell Lung Cancer	USA	Prebieha
REO 015	Fáza II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Advanced Head and Neck Cancers	USA	Prebieha
REO 014	Fáza II	Intravenous Administration of REOLYSIN® for Patients with Metastatic SarcomasIntravenous Administration of REOLYSIN® for Patients with Metastatic Sarcomas	USA	Ukončená
REO 013	Translačná Štúdia	Intravenous Administration of REOLYSIN® for Patients with Metastatic Colorectal CancerIntravenous Administration of REOLYSIN® for Patients with Metastatic Colorectal Cancer	VB	Prebieha
MAYO-MC0672 (NCI Trial)	Fáza II	Intravenous Administration of REOLYSIN® for Patients with Metastatic Melanoma	USA	Ukončená
OSU-07022 (NCI Trial)	Fáza I/II	Systemic and Intraperitoneal Administration of REOLYSIN® for Patients with Metastatic Ovarian, Peritoneal and Fallopian Tube CancersSystemic and Intraperitoneal Administration of REOLYSIN® for Patients with Metastatic Ovarian, Peritoneal and Fallopian Tube Cancers	USA	Prebieha
REO 012	Fáza I	Intravenous Administration of REOLYSIN® in Combination with Cyclophosphamide for Patients with Advanced Malignancies	VB	Prebieha

Číslo štúdie	Fáza	Názov štúdie	Miesto	Status
REO 011	Fáza I/II	Intravenous Administration of REOLYSIN® in Combination with Paclitaxel and Carboplatin for Patients with Advanced Head and Neck Cancers	VB	Ukončená
REO 010	Fáza I	Intravenous Administration of REOLYSIN® in Combination with Docetaxel for Patients with Advanced Malignancies	VB	Ukončená
REO 009	Fáza I	Intravenous Administration of REOLYSIN® in Combination with Gemcitabine for Patients with Advanced Malignancies	VB	Ukončená
REO 008	Fáza II	Intratumoral Administration of REOLYSIN® in Combination with Low-Dose Radiation for Patients with Advanced Malignancies	VB	Ukončená
REO 007	Fáza I/II	Infusion Monotherapy of REOLYSIN® for Patients with Recurrent Malignant Gliomas	USA	Ukončená
REO 006	Fáza I	Local Administration of REOLYSIN® in Combination with Radiation for Patients with Advanced Cancers	VB	Ukončená
REO 005	Fáza I	Systemic Administration of REOLYSIN® for Patients with Various Metastatic Tumors	VB	Ukončená
REO 004	Fáza I	Systemic Administration of REOLYSIN® for Patients with Various Metastatic Tumors	USA	Ukončená
REO 001	Fáza I	Local Monotherapy of REOLYSIN® for Patients with Subcutaneous Tumors	Kanada	Ukončená
REO 003	Fáza I/II	Local Monotherapy of REOLYSIN® for Patients with Recurrent Malignant Gliomas	Kanada	Ukončená
REO 002	Translačná Štúdia	Local Monotherapy of REOLYSIN® for Patients with T2 Prostate Cancer	Kanada	Ukončená

Tabuľka č. 2. Prehľad klinických štúdií vedených firmou Oncolytics Biotech® Inc. s REOLYSIN®-om. Stav v septembri 2012. Upravené podľa Oncolytics Biotech® Inc., Copyright Oncolytics Biotech® Inc.

2.3 HYPOXIA

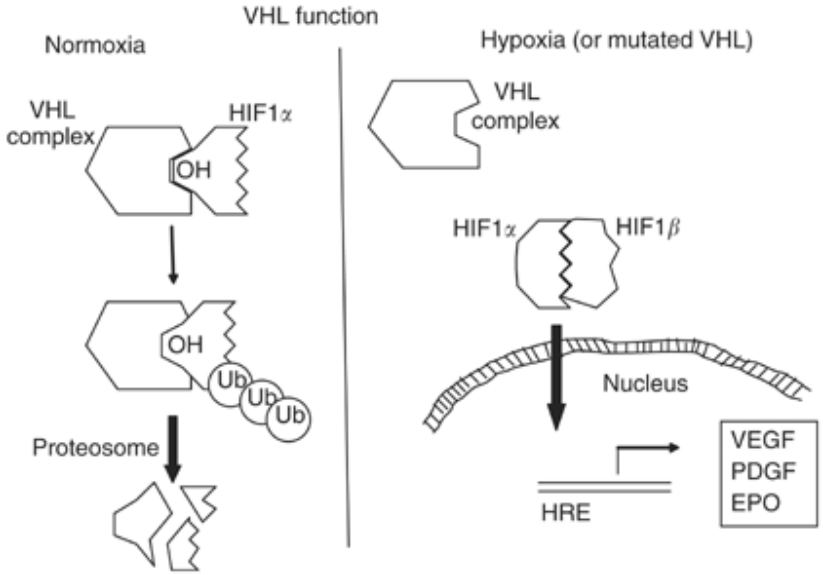
2.3.1 HYPOXIA A NÁDOR

Hypoxia je jav, ktorý nastáva v značnom počte ľudských ochorení zahŕňajúcich ischémiu myokardu, kritickú ischémiu končatín, mozgovú mŕtvicu a nádorové ochorenia. (Semenza G.L. et al., 2000) U pacientov s kritickou ischémiou končatín hypoxia a s ňou asociované poškodenie tkaniva môžu vyústiť v amputáciu (Niinikoski J., 2003). Rozľahlá hypoxia u pacientov po mozgovej mŕtvici môže spôsobiť bunkovú smrť i za hranicami iniciálneho poškodenia (Zauner A. et al., 2002). Jej výskyt v nádorových ochoreniach je často prognostickým faktorom slabšieho prežívania pacientov a pokladá sa za selektívny u vysoko agresívnych nádorov (Brown J.M., 1999, Wouters B.G. et al., 2002). Navyše, hypoxia sa prednostne vyskytuje v bunkách s mutovaným p53 a v bunkách s defektmi v apoptóze (Graeber T.G. et al., 1996) a taktiež podporuje angiogenézu potrebnú pre kontinuálny rast nádorových buniek v malígnych podmienkach (Brown J.M. a Giaccia A.J., 1998, Wouters B.G. et al., 2003). Normálne tkanivo má parciálny tlak kyslíka 50-60 mm Hg, zatiaľ čo väčšina solídnych nádorov ho má 10 mm Hg a menej (Brown J.M. a Wilson W.R., 2004, Hockel M. a Vaupel P., 2001b). Hypoxické prostredie indukuje adaptívne zmeny v metabolizme nádorových buniek, čo môže úplne zmeniť lokálne mikroprostredie. Tieto zmeny sú klinicky dôležité, keďže hypoxia zosiľňuje rezistenciu k chemoterapii a rádioterapii (Hockel M. a Vaupel P., 2001b), zvyšuje schopnosť buniek metastázovať a zvyšuje nestabilitu genómu (Hockel M. a Vaupel P., 2001a) Práve preto je hypoxia dôležitou súčasťou výskumu v týchto ochoreniach a úspešný, na vírusoch podložený prístup, by v budúcnosti mohol prispieť k zlepšeniu liečebných výsledkov.

2.3.2 MOLEKULÁRNY MECHANIZMUS ADAPTÁCIE NA HYPOXIU

Transkripčné faktory, členy rodiny hypoxiou indukovaného faktoru (HIF), sú kľúčovými regulátormi adaptívnej bunkovej odpovede na hypoxiu. Jedným z hlavných modulátorov odpovede nádorovej bunky na hypoxiu je HIF-1 (viď obrázok 5). HIF-1 existuje ako heterodimér HIF-1α a HIF-1β (taktiež známy ako aryl hydrokarbón receptor jadrový translokátor, ARNT). Zatiaľčo HIF-1β je konštitutívne exprimovaný, hladina HIF-1α je za normálnych okolností

udržiavaná nízka pomocou proteozomálnej degradácie. Nadmerná expresia HIF-1α je charakteristická pre mnoho rozličných nádorov a jeho konštitutívna aktivácia je často pozorovaná v nádoroch s agresívnym fenotypom (Bardos J.I. a Ashcroft M., 2004). HIF-1α je degradovaný na proteazóme závislou cestou mediovanou ubikvitináciou v normoxických podmienkach (Jaakkola P. et al., 2001) Na kyslíku závislá premena HIF-1α je regulovaná prolyl HIF hydroxylačnými (PHD) enzýmami, ktoré hydroxylujú dva konzervované prolínové zvyšky umiestnené v na kyslíku závislej degradačnej doméne HIF-1α. V normoxii, hydroxylácia HIF-1α pomocou enzýmov PHD dovolí jeho naviazanie na von Hippel Lindau (VHL) proteín, rozpoznávací komponent E3 ubikvitín ligázového komplexu. Táto interakcia podnieti ubikvitináciu HIF-1α, ktorá je mediovaná komplexom, ktorý pozostáva z VHL, elongin-B, elongin-C, Cullin 2 a Rbx1 a následne vedie k degradácii HIF-1α. (Maxwell P.H. et al., 1999, Tanimoto K. et al., 2000). Navyše, na kyslíku závislá hydroxylácia HIF-1α enzýmovým faktorom inhibujúcim HIF-1 blokuje interakciu HIF-1α s transkripčným koaktivátorom p300/CBP, a preto inhibuje HIF-1 mediovanú génovú transkripciu v normoxických podmienkach (Jeong J.W. et al., 2002). V hypoxických podmienkach sa rýchlosť hydroxylácie asparagínu a prolínu znižuje, VHL sa nemôže viac naviazať na HIF-1α čo vedie k stabilizácii proteínu. Stabilizovaný HIF-1α translokuje do jadra kde interaguje s transkripčnými koaktivátormi HIF-1β a p300/CBP (Jaakkola P. et al., 2001) a tým transaktivuje radu cieľových génov viď tabuľka č.3 (Mahon P.C. et al., 2001, Semenza G.L., 2000, Semenza G.L. et al., 2000).



Obrázok č. 5. VHL a HIF-1 dráhy.

VHL komplex (zložený z von Hippel-Lindau proteínu, elongínu B, elongínu C, Cul2, a Rbx1) pôsobí na reguláciu hladiny hypoxiou-indukovateľného faktora HIF-1α. Počas normoxie je HIF-1α hydroxylovaný na dvoch prolínových zvyškoch na kyslíku závislým enzymatickým mechanizmom. VHL komplex sa naviaže k hydroxylovanému HIF-1α čo vedie k proteozómom sprostredkovanej degradácii HIF-1α. Počas hypoxie nie je HIF-1α hydroxylovaný, a preto nemôže naviazať VHL komplex. HIF-1α akumuluje a viaže sa na HIF-1β čím vytvoria HIF-1 komplex, ktorý následne translokuje do bunkového jadra, kde sa viaže s hypoxia-responsive element (HRE) v génových promotoroch a umožňuje expresiu hypoxiou-indukovaných génov. Podobne, strata funkčných mutácií VHL zabraňuje ubiquitín-mediovanej degradácii HIF-1α , čím sa zvýši expresia hypoxiou-indukovaných génov.

Funkcia	Gén
Erytropoéza/ metabolizmus železa	Erytropoietin (EPO) Transferrin (Tf) Transferrin receptro (Tfr) Ceruloplazmín
Angiogenéza	Vascular endotelial growth factor (VEGF) Endocrine-gland-derived VEGF(EG-VEGF) Leptin (LEP) Transforming growth factor β3 (TGF-β3)
Cievny tonus	Nitric oxide synthase (NOS2) Heme oxygenase 1 Endothelin 1 (ET1) Adrenomedulin (ADM) A_{1B} Adrenergic receptor
Metabolizmus v matrix	Matrix metalloproteinases (MMPs) Plasminogen activator receptors and inhibitors (PAIs) Collagen prolyl hydroxylase
Glukózový metabolizmus	Adenylate kinase-3 Aldolase A, C (ALDA, C) Carbonic anhydrase-9 Enolase-1 (ENO1) Glucose transporter 1, 3 (GLUT 1, 3) Glyceraldehyde phosphate dehydrogenase (GAPDH) Hexokinase 1,2 (HK,2) Lactate dehydrogenase-A (LDHA) Pyruvate kinase M (PKM) Phosphofructokinase (PFKL) Phosphoglycerate kinase 1 (PGK 1) 6-phosphofructo-2-kinase/fructose-2,6-biphosphate-3 (PFKFB-3)
Bunková proliferácia/ prežívanie	Insuline like growth factor-2 (IGF II) Transforming growth factor-α (TGFα) Adrenomodullin (ADM)
Apoptóza	Bcl-2 /adenovirus E1B19kDa-interacting protein 3 (BNIP3) BNIP 3 like protein X (NIX)

Tabuľka č.3 Najdôležitejšie cieľové gény HIF-1α proteínu. Spracované podľa Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. Exp Mol Med. 2004;36(1):1-12, Otrock ZK, Hatoum HA, Awada AH, Ishak RS, Shamseddine AI.Hypoxia-inducible factor in cancer angiogenesis: structure, regulation and clinical perspectives. Crit Rev Oncol Hematol. 2009;70(2):93-102 a Patiar S, Harris AL.Role of hypoxia-inducible factor-1alpha as a cancer therapy target. Endocr Relat Cancer. 2006;13 Suppl 1:S61-75.

HIF-1 teda hrá dôležitú úlohu v ochrane solídnych nádorov proti hypoxii podnecovaním angiogenézy, indukovaním expresie rastových faktorov, prevenciou apoptózy alebo zvyšovaním anaeróbneho metabolizmu. Zvýšená expresia HIF-1α bola spozorovaná v mnohých ľudských nádoroch a koreluje so zlou prgnózou a rezistenciou k rádio- a chemoterapii (Jaakkola P. et al., 2001, Semenza G.L. et al., 2000, Zhong H. et al., 1999).

2.3.3 HIF-1A APOPTÓZA

Napriek tomu, že HIF-1α zvyčajne participuje v adaptínych odpovediach v hypoxii na podnecovaní nádorového prežitia, určitá rola HIF-1α v regulácii apoptózy tiež nie je nepodstatná. HIF-1α podporuje bunkovú smrť zvyšovaním p53 alebo ďalších proapoptotických proteínov ako napríklad Bcl-2/E1B 19kDa interagujúceho proteínu 3 (BNIP3) alebo BNIP3L (NIX) (Bruick R.K., 2000, Carmeliet P. et al., 1998, Guo K. et al., 2001, Sowter H.M. et al., 2001). V najčastejšom prípade však HIF-1α podporuje prežitie nádorových alebo endoteliálnych buniek v hypoxických podmienkach a takisto chráni pred apoptózou indukovanou depriváciou séra alebo spôsobenou protinádorovými látkami (Alvarez-Tejado M. et al., 2001, Kim J.Y. et al., 2004, Piret J.P. et al., 2004, Sasabe E. et al., 2005, Zhang Q. et al., 2004). HIF-1α uplatňuje svoju protiapoptotickú funkciu transkripčnou aktiváciou antiapoptotických proteínov, napríklad z rodiny Bcl-2 alebo inhibítorov apoptózy IAP, alebo alternáciou v bunkovom energetickom metabolizme znížením glukózovej absorpcie a glykolýzy (Blum R. et al., 2005, Dong Z. et al., 2003, Jaakkola P. et al., 2001, Mathupala S.P. et al., 2001, Park S.Y. et al., 2002, Zhang Q. et al., 2004).

2.3.4 HYPOXIA A VÍRUSOVÁ INFEKCIA

Biologické vlastnosti onkolytických vírusov, hlavne ich schopnosť replikácie vnútri nádorovej bunky a neskoršie šírenie z bunky na bunku, sú pre nádorovú terapiu vysoko žiaduce charakteristiky. Keďže hypoxia je dôležitou vlastnosťou solídnych nádorov, práve schopnosť vírusov replikovať sa v hypoxickom prostredí môže byť jedným z kritických determinantov v úspechu alebo v zlyhaní vírusovej terapie. Vypnutie translácie proteínov je základným

procesom v bunkovej adaptácii na mnoho druhov stresu, vírusovú infekciu a hypoxiu nevynímajúc. Ako sa špecifický vírus dokáže vyrovnať s bunkovými mechanizmami, ktoré regulujú transláciu v bunke v stave stresu, môže byť kritické pre úspech vírusovej terapie.

Hypoxia predstavuje hlavnú prekážku, ktorej sa musí nádorová bunka prispôsobiť, aby si zabezpečila prežitie (Harris A.L., 2002). Hypoxia spôsobuje blok bunkového cyklu v G_1-fáze bunkového cyklu v netransformovaných bunkách (Gardner L.B. et al., 2001, Gardner L.B. et al., 2003, Goda N. et al., 2003), zatiaľ čo mnohé vírusy potrebujú mať bunku v S- fáze alebo indukujú S-fázu k odblokovaniu svojej replikácie. Aj keď G_1-blok je pozorovaný v normálnych bunkách v hypoxických podmienkach, bunky s narušenou HIF-1α signálnou dráhou a niektoré nádorové bunky v hypoxii prechádzajú voľne bunkovým cyklom alebo je u nich nájdená zvýšená S fáza (Goda N. et al., 2003, Pipiya T. et al., 2005). G_1-blok nádorových buniek v hypoxických podmienkach preto zrejme nebude rozhodujúci v limitovaní vírusovej replikácie. DNA replikácia môže byť v hypoxii blokovaná, ale len pri anoxii <0,1% kyslíka.

Vírusová infekcia je takisto veľmi škodlivá pre bunku a v normálnej bunke sa okamžite zapínajú obranné mechanizmy na predchádzanie vírusovej infekcie po vniknutí vírusu do bunky. Eventuálny úspech alebo zlyhanie vírusovej replikácie v nádorovej bunke môže byť preto závislé na vzájomnej súhre medzi adaptívnymi procesmi, ktoré umožňujú bunke prežiť v hypoxických podmienkach a bunkových obranných odpovediach, ktoré bránia vírusovej replikácii. Hlavná bunková odpoveď na stresové podnety, ku ktorým patrí hypoxia i vírusová infekcia, je vypnutie proteosyntézy. Keďže translácia virálnych proteínov je závislá na prvkoch translačnej mašinérie hostiteľa, schopnosť translácie bunkových i virálnych proteínov počas transformácie, infekcie a hypoxie môže byť rozhodujúca pre úspech či zlyhanie viroterapie.

Všeobecne sa vie veľmi málo o replikácii vírusov v hypoxických podmienkach. Napriek tomu sa nájde pár novších článkov popisujúcich dopad hypoxie na replikáciu niektorých vírusov a niektoré z nich sa dokonca zaoberali onkolytickými vírusmi.

2.3.4.1 Adenovírusy

Najviac klinických štúdií s replikujúcimi sa vírusmi bolo vedených práve s adenovírusmi, ale napriek biologickej výhode cielenej replikácie boli výsledky klinických štúdií sklamaním (Hawkins L.K. et al., 2002, Heise C. a Kirn D.H., 2000). Navyše, wild-

type adenovírus je v pokusoch na zvieracích modeloch málokedy schopný eradikovať nádor po podaní jednorázovej intratumorálnej dávky (Doronin K. et al., 2000, Harrison D. et al., 2001). Napriek zlyhaniu eliminácie nádorových xenoimplantátov, vysoké vírusové titre boli nerovnomerne distribuované v nádoroch ešte niekoľko týždňov po jedinej vírusovej injekcii (Harrison D. et al., 2001, Sauthoff H. et al., 2003). Pozorovania tejto nádorovej perzistencie, napriek vysokým dávkam vírusu prítomného v nádore, naznačujú limitácie v rozširovanie vírusu v nádoroch a to môže obmedzovať terapeutický efekt replikujúcich sa adenovírusov.

Mnohé fyzikálne bariéry v organizácii nádoru môžu potenciálne blokovať rozšírenie adenovírusu. Extracelulárna matrix je jedna z možností. Adenovirálna infekcia je primárne mediovaná interakciou vírusu s coxackie-adenovírusovým receptorom (CAR) a integrínovým receptorom na povrchu bunky (Roelvink P.W. et al., 1998). Keďže expresia CAR je vysoko variabilná v rôznych nádoroch (Kim M. et al., 2002), práve znížená expresia tohto receptoru v nádore môže predstavovať potenciálnu bariéru v replikácii a šírení adenovírusu.

Takisto existujú dôkazy, že práve hypoxia redukuje replikáciu adenovírusov. V xenoimplantátoch nádorov boli bunky infikované adenovírusom topograficky asociované s krvnými cievami (Sauthoff H. et al., 2003). Tento objav, spolu s pozorovaním, že povrchové epitélie, ktoré sú prirodzene osídlené adenovírusmi, epitélium dýchacích ciest a spojovky, sú vystavené okolitým a preto normoxickým hladinám, poukazuje na inklináciu adenovírusu k tkanivám s vyšším parciálnym tlakom kyslíka (Pipiya T. et al., 2005).

Dve štúdie popísali podstatný pokles v produkcii vírusu v niekoľkých nádorových líniách v hypoxických podmienkach (Pipiya T. et al., 2005, Shen B.H. a Hermiston T.W., 2005). Použitá hladina hypoxie (cca 1% O_2, 7,5mmHg) bola podobná hladine nameranej v nádore. Hypoxia nemala efekt na expresiu hladiny CAR ani integrínových proteínov, na ktoré sa vírus viaže, ani efekt na up-take vírusu (Shen B.H. a Hermiston T.W., 2005) ani vplyv na transkripciu vírusových génov (Pipiya T. et al., 2005, Shen B.H. a Hermiston T.W., 2005). Napriek tomu bola translácia vírusových proteínov markantne znížená (Pipiya T. et al., 2005).

Zníženie translácie virálnych proteínov v hypoxických podmienkach vzbudzuje veľký záujem, pretože adenovírus adaptoval veľa mechanizmov na obídenie vypnutia translácie ako bunkovej odpovede na vírusovú infekciu. Adenovírusy, expresiou VA RNA môžu obísť PKR-mediovaný translačný blok, ktorý je stimulovaný IFN a dsRNA a mediovaný fosforyláciou eIF2α

(Schneider R.J. a Mohr I., 2003). Navyše, adenovírus si sám vypína transláciu hostiteľských preotínov v neskorej fáze jeho infekčného cyklu defosforyláciou eIF4E čiapočku-viažucim proteínom. Neskoré virálne proteíny môžu byť stále prekladané, pretože neskoré virálne mRNA obsahujú trojdielnu prednú časť, ktorá využíva modifikovaný čiapočku-viažuci komplex obsahujúci vírusový 100kDa proteín (Cuesta R. et al., 2004). Dráha, ktorou hypoxia limituje transláciu ostáva neznáma, ale môže zahŕňať mTOR dráhu alebo prvky nasledujúce po eIF2α.

2.3.4.2 Vírus vezikulárnej stomatitídy

Vírus vezikulárnej stomatitídy je nádejným vektorom pre génovú terapiu, pretože jeho replikácia sa zdá byť prirodzene namierená na nádorové bunky. Normálne myšacie embryonálne fibroblasty sú obyčajne rezistentné voči infekcii VSV, čo je sčasti spôsobené dsRNA-mediovanou aktiváciou PKR po vstupe vírusu a vypnutí translácie. Avšak po transformácii fibroblastov sa tieto stávajú vysoko citlivé na VSV infekciu. (Balachandran S. a Barber G.N., 2004). Tento účinok sa zdá byť následkom oslabených antivirálnych účinkov inteferónu v transformovaných bunkách. Prekvapivo, aktivácia PKR a fosforylácia eIF2α je v normále. Avšak, eIF2B-mediovaná guanín-nukloetidová výmenná aktivita v dráhe ležiacej pod eIF2 je často aberantná v transformovaných bunkách, premáhajúca účinky fosforylácie eIF2α a povoľujúca transláciu VSV mRNA.

VSV mRNA expresia je zvýšená v hypoxických podmienkach a iniciálna supresia translácie vo včasných fázach je potlačená a nastáva abundantná syntéza virálnych proteínov napriek fosforylácii eIF2α a defosforylácii eIF4E (Connor J.H. et al., 2004).

2.3.4.3 Herpes simplex vírus

HSV má veľa želaných vlastností vhodných na prípravu replikačného vektoru génovej terapie. Zatiaľ nie sú jasné dáta o účinkoch hypoxie na replikáciu HSV. Je známa iba jedna štúdia, ktorá použila hypoxiu na nabudenie expresie ribonukleotid reduktázového génu a dokázala, že HSV s chýbajúcim ribonukleotid reduktázovým génom je schopný replikácie v hypoxických podmienkach (Pin R.H. et al., 2004).

2.3.4.4 Reovírus

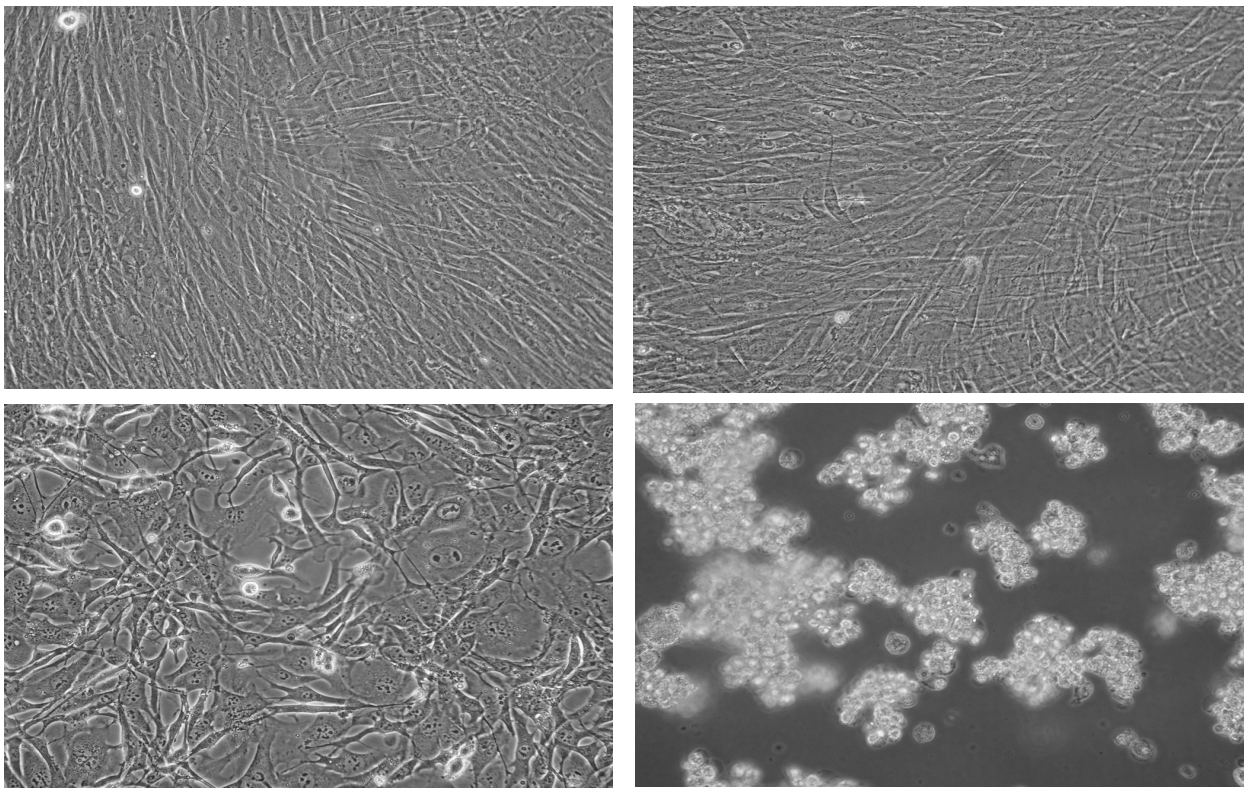
O schopnosti replikácie reovírusu v hypoxických podmienkach doteraz pojednáva jediná štúdia. Autori zistili, že reovirálna infekcia v hypoxii potláča HIF-1α na proteínovej úrovni v bunkách nádoru kolonu HCT116. Reovirálna infekcia bola schopná redukovať hladiny HIF-1α v oboch von Hippel Lindau (VHL) -/- A498 bunkách (renálny karcinóm) i v p53 -/-HCT116 bunkách, z čoho vyplýva, že zníženie HIF-1α spôsobené reovírusom nepotrebuje VHL ani p53 preoteín. V ďalších pokusoch zistili, že toto indukované zníženie HIF-1α proteínu je závislé na aktivite proteozómu a bunky s konštitutívnou expresiou HIF-1α boli relatívne rezistentné voči apoptóze indukovanej reovírusom (Cho I.R. et al., 2010).

3. Ciele

V našej práci sme sa zamerali na štúdium biologických aspektov účinku reovírusu na nádorové bunky v systémoch *in vitro* i *in vivo*. Sledovali sme schopnosť reovírusu infikovať a zabíjať nádorové i nenádorové bunky. Posudzovali sme schopnosť reovírusu indukovať protinádorovú imunitu za použitia modelu *in vivo*. Zisťovali sme protinádorovú účinnosť reovírusu v hypoxii a takisto mechanizmy bunkovej smrti, ktorý vírus vo svojom hostiteľovi vyvoláva. Takmer všetky výsledky a ich diskusie sú podrobne spracované v štyroch publikáciách, ktoré sú priložené. V predloženej práci uvádzam prehľad hlavných výsledkov a ich stručnú diskusiu.

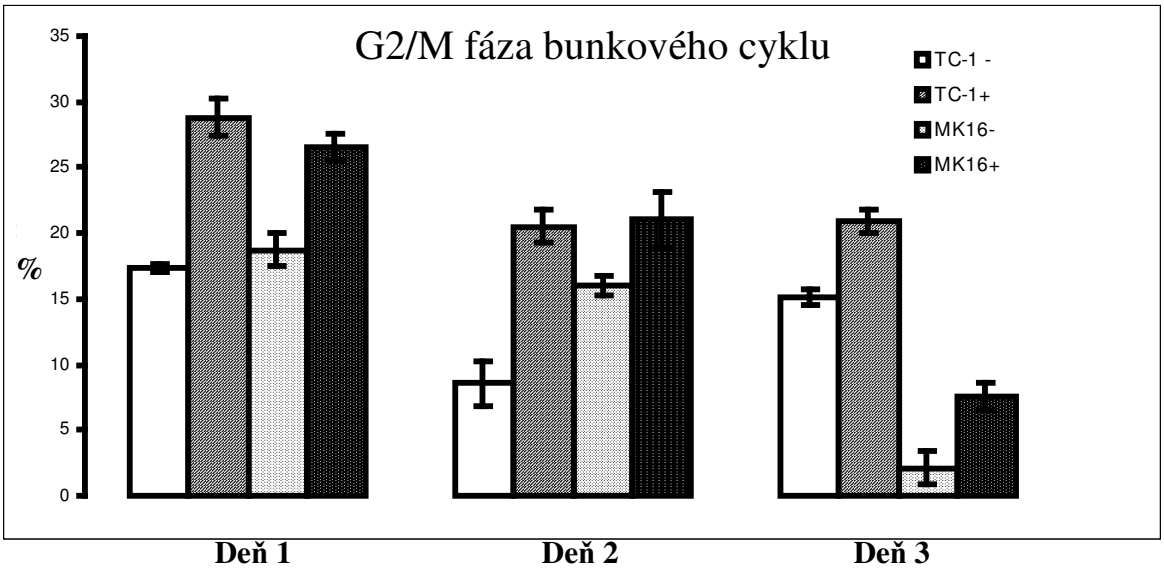
4. Výsledky

Na bunkových líniách TC-1 a MK16/III/ABC (MK16), ktoré boli podrobne popísané inde (Sobotkova E. et al., 2008) sme skúmali nielen priamy onkolytický efekt reovírusu, ale takisto jeho schopnosť vyvolať protinádorovú imunitnú odpoveď proti solídnym nádorom na modeli myšacieho transplantátu a taktiež patogenicitu reovírusom-infikovaných buniek. Obidve línie sú vysoko citlivé voči reovírusu a produkovali značné množstvo infekčných partikulí *in vitro*. Napriek tomu sa tieto línie v účinnosti líšili. TC-1 bunky produkovali o niečo menej infekčného vírusu, ale, paradoxne, boli viac účinné v produkcii σ1 reovirálneho antigénu a ako dôsledok vírusovej infekcie umierali skôr a radikálnejšie ako simultánne infikované MK16 bunky. Ako pozitívnu kontrolu sme používali normálne ľudské fibroblasty, v ktorých sa reovírus nereplikuje, a ktoré preto nezabíja.

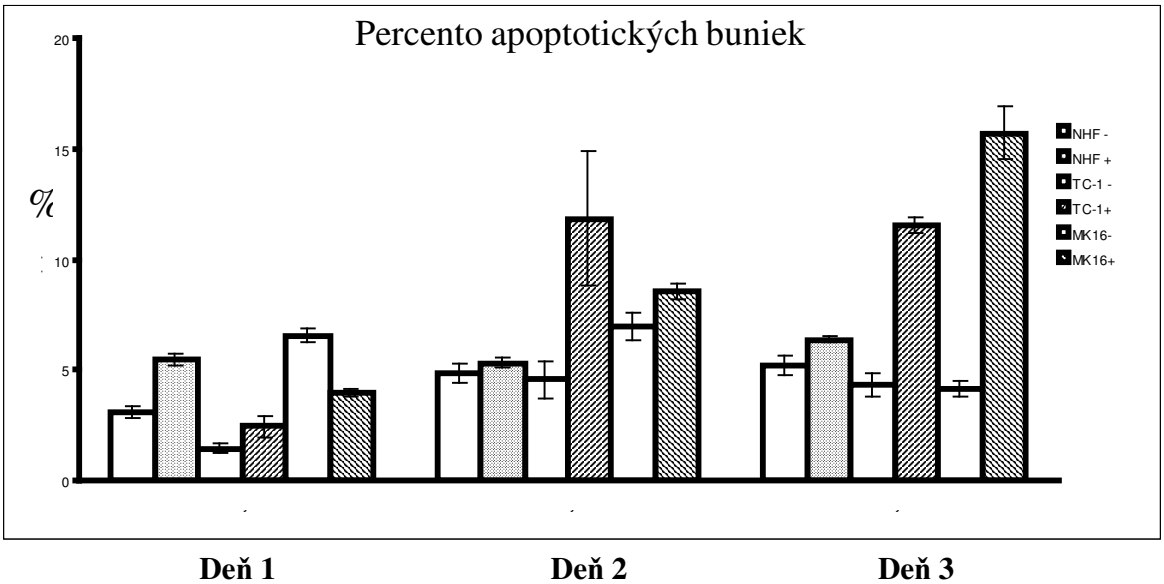


Obrázok 6. Účinok reovírusu na bunky. Hore bunková línia fibroblastov, dolu bunková línia TC-1. Vľavo neinfikovaná kontrola, vpravo 96 hod po infekcii reovírusom (MOI=10), zväčšenie 200x.

Malé rozdiely medzi líniami boli pozorované i v percentuálnom zastúpení buniek zastavených v G2/M fáze bunkového cyklu a v niektorých apoptotických markeroch. Graf č. 1 a 2 názorne zobrazuje namerané výsledky.

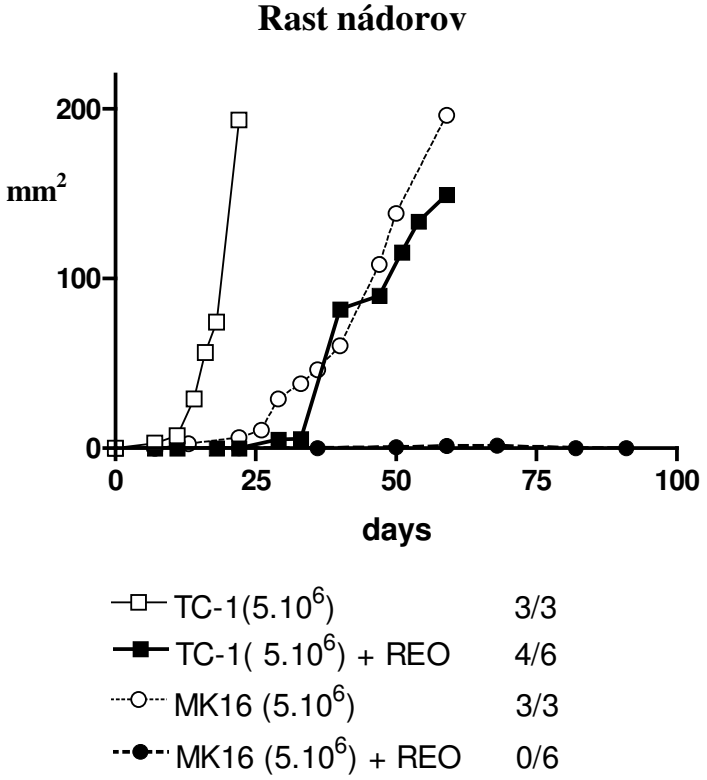


Graf č. 1 G2/M fáza bunkového cyklu skúmaných línií po infikovaní a bez infekcie reovírusom.



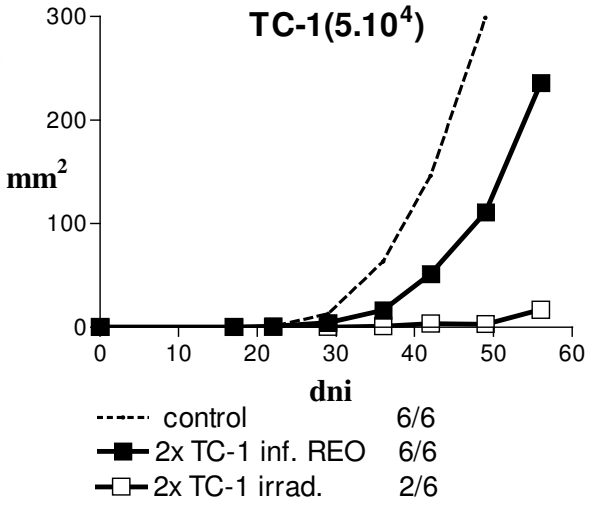
Graf č. 2 Namerané percento apoptózy v skúmaných bunkových líniách po a bez infekcie reovírusom.

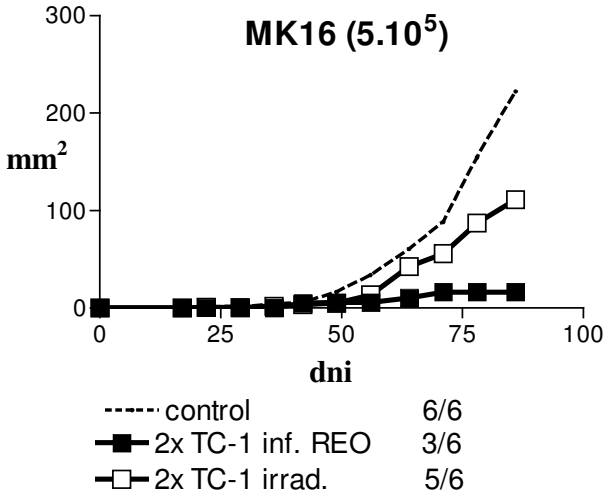
Vplyv reovírusu na životnosť oboch línií sa manifestoval zníženou onkogénnou schopnosťou línií v myšasom modeli. Keď sme naočkovali vysoké dávky ($5x10^6$) reovírusom infikovaných buniek (MOI 10 PFU/bunku) do imunologicky kompatibilných zvierat, ich onkogénna aktivita bola silne suprimovaná, takmer úplne v prípade MK 16 buniek a o niečo menej v prípade viac onkogénnych TC-1 buniek. Výsledky zobrazuje graf č.3.



Graf č.3 Rast nádorov reovírusom infikovaných a neinfikovaných TC-1 a MK16 buniek a výskyt nádorov/ počet experimentálnych zvierat.

Najdôležitejším cieľom tejto štúdie bolo zistiť, či použité onkogénne bunkové línie po infekcii reovírusom budú predstavovať silnejší imunogén než samotné neinfikované ožiarené bunky. V imunizačných experimentoch boli použité TC-1 bunky a obidve línie MK 16 a TC-1 slúžili ako čelenž. Imunizačné experimenty, v ktorých neonkogénna dávka (10^6) reovírusom infikovaných TC-1 buniek bola podávaná paralelne s rovnakými dávkami ožiarených buniek priniesli neočakávané výsledky. Keď boli imunizované zvieratá čelenžované TC-1 bunkami, ožiarené bunky boli dokázateľne lepším imunogénom než infikované bunky. Navyše, pri MK 16 bunkách opak bol pravdou: imunita indukovaná infikovanými TC-1 bunkami sa zdá byť silnejšia než tá spôsobená ožiarenými bunkami. Výsledky zobrazuje graf č.4A a 4B.



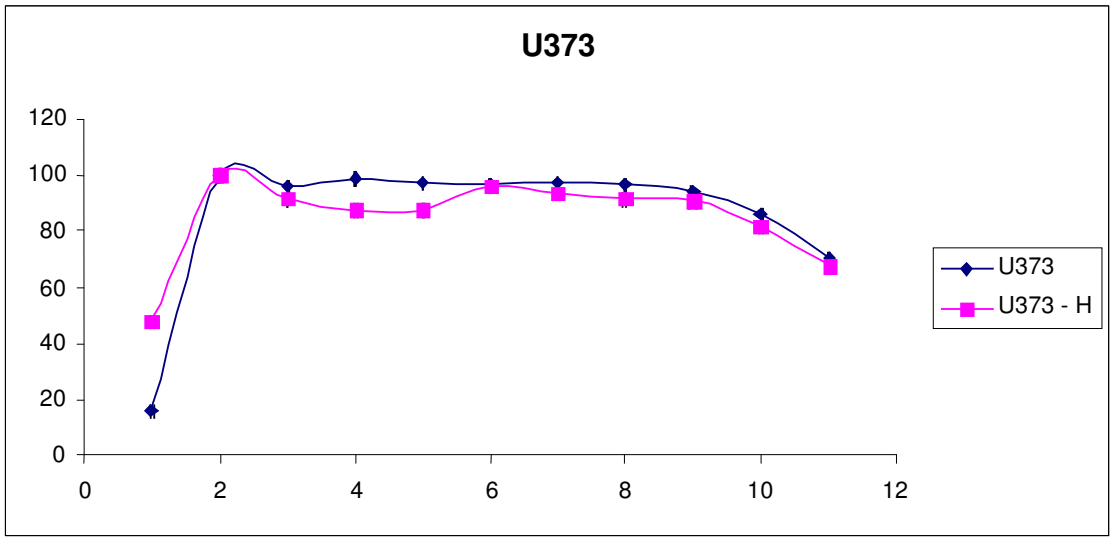


Graf č.4A. Rast nádorov v imunizovaných a neimunizovaných myšiach po čelenži TC-1 bunkami.
2x TC-1 inf. REO *vs.* neimunizované (TC-1) $p<0.02$
2x TC-1 irrad. vs. neimunizované (TC-1) $p<0.001$
2x TC-1 inf. REO *vs*. 2x TC-1 ožiarené $p<0.001$

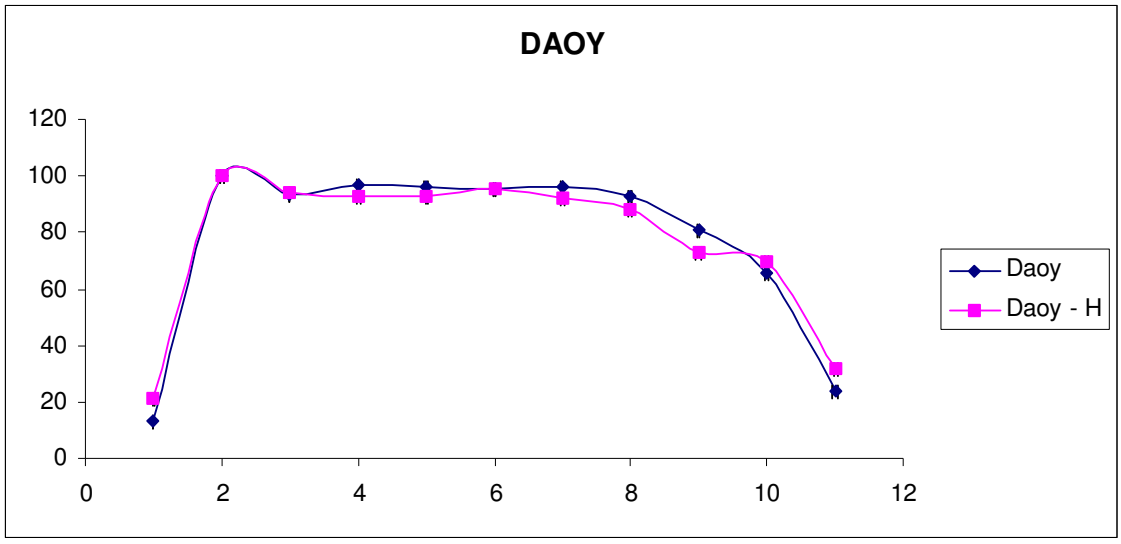
Graf č.4B. Rast nádorov v imunizovaných a neimunizovaných myšiach po čelenži MK 16 bunkami.
2x TC-1 inf. REO *vs.* neimunizované $p<0.0001$
2x TC-1 irrad. *vs*. neimunizované $p<0.02$
2xTC-1+ REO *vs* 2x TC-1 ožiarené $p<0.01$

Dôvody týchto rozdielov dodnes nepoznáme, predpokládáme však, že sú výsledkom rozdielnosti skúmaných bunkových línií. Výsledky sme publikovali v pôvodnom článku v časopise *Neoplasma* roku 2010.

V ďalšej sérii experimentov sme skúmali schopnosť reovírusu replikovať sa a vyvolávať onkolytický účinok v bunkách v normoxických i v hypoxických podmienkach. V našich experimenoch sme používali meduloblastómovú bunkovú líniu a líniu ľudského glioblastómu, Daoy a U373, v porovnaní s kontrolnou netransformovanou líniou ľudských fibroblastov. Hypoxiu sme indukovali kultiváciou buniek v hypoxickej komôrke (Billups-Rothenberg, Inc., Del Mar, CA, USA) v atmosfére presne namiešanej zmesi plynov s obsahom 1% kyslíka, 5% oxidu uhličitého a 94% dusíku. Dokázali sme, že reovírus je nielen schopný replikácie i v hypoxickom nádorovom mikroprostredí, ale taktiež je schopný spôsobiť bunkovú smrť svojho hostiteľa. Úmrtnosť buniek sme merali MTT testom, ktorý je založený na dôkaze zmien viability meranej na základe mitochondriálneho oxidačného mechanizmu živých buniek. Výsledky priemerov naších meraní zobrazujú grafy jednotlivých línií č.5 6, z ktorých vidieť, že schopnosť reovírusu zabíjať nádorové bunky v hypoxii je oproti normoxii neoslabená.

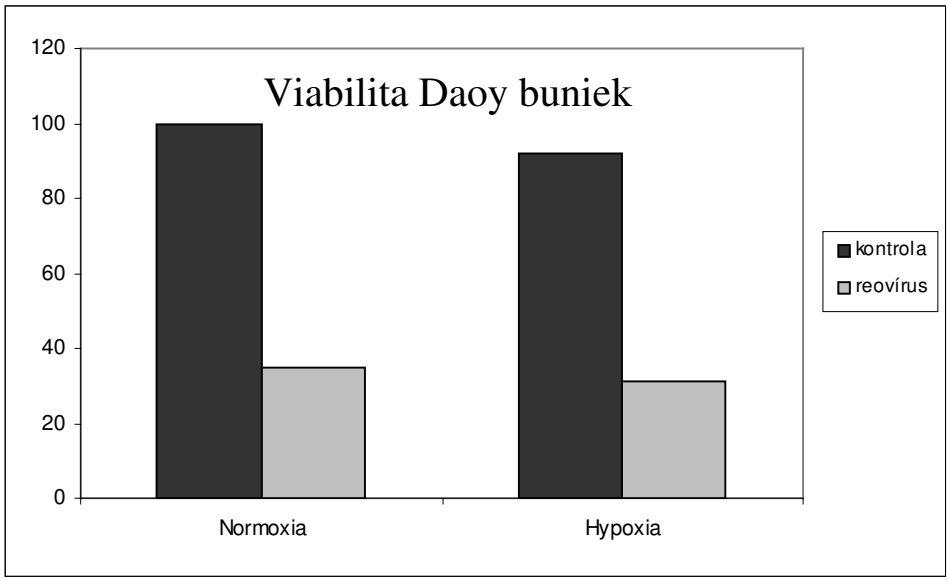


Graf č.5 Percento prežitia U373 buniek po infekcii reovírusom v normoxii a v hypoxii.

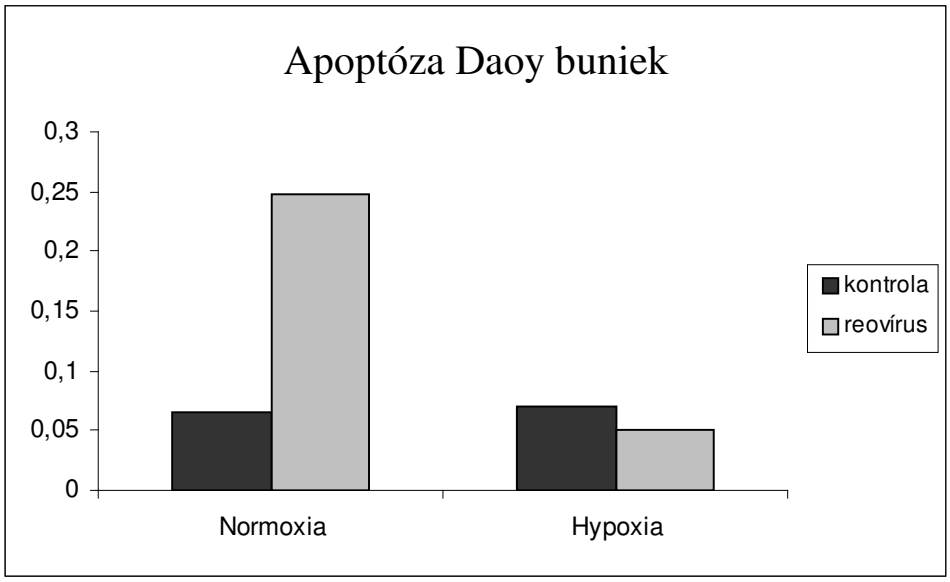


Graf č.6 Percento prežitia Daoy buniek po infekcii reovírusom v normoxii a v hypoxii.

Po zistení, že reovírus je schopný zabíjať nádorové bunky i v hypoxii sme zisťovali aké je percento apoptotických buniek v týchto vzorkách. Zistili sme, že nepomerne viac buniek umiera apoptózou v normoxii. Graf č.7A a 7B.

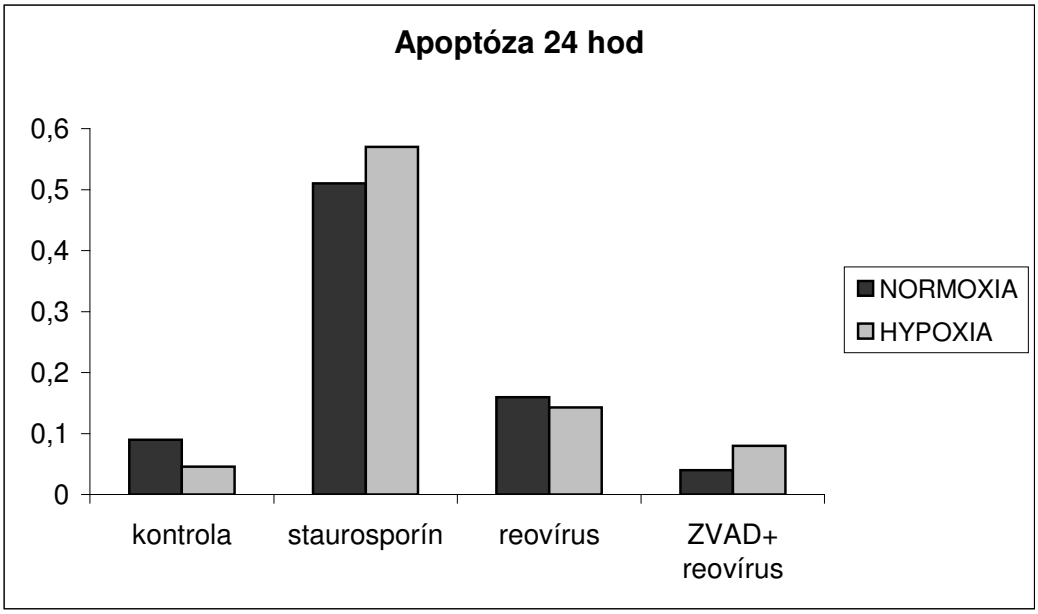


Graf č.7A Viabilita Daoy buniek po infekcii reovírusom 24 hodín postinfekčne.
Viabilné sú bunky, které neviažu AnexinV ani do nich nevstupuje propídium jodid.



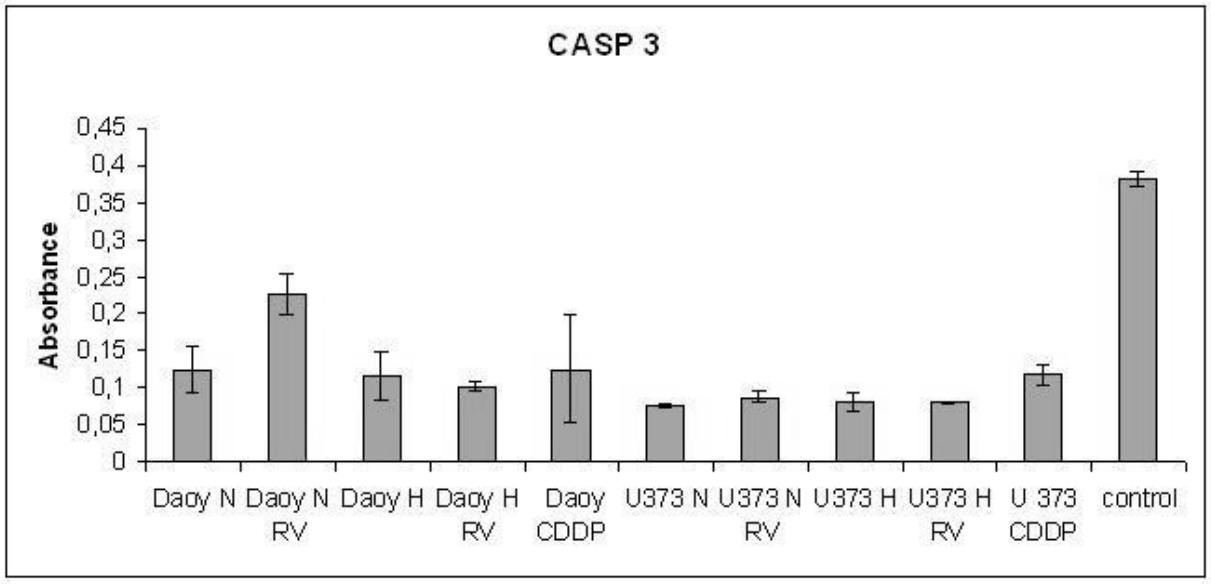
Graf č.7B. Apoptóza Daoy buniek po infekcii reovírusom 24 hodín postinfekčne.
Apoptotické sú bunky viažuce anexin V.

Na overenie našich zistení, sme urobili sériu pokusov s pan-kaspázovým inhibítorom, ZVAD-fmk. Tieto preukázali, že po pridaní inhibítora pred podaním reovírusu, sa hladiny apoptózy v normoxii a hypoxii dramaticky menia. ZVAD-fmk zablokoval hladinu apoptózy v normoxických bunkách úplne, zatiaľčo v hypoxických nie. Z toho vyplýva, že v hypoxii prebieha apoptóza nezávislá na kaspázach. Graf č. 8



Graf č. 8. Apoptóza u Daoy buniek po zablokovaní kaspáz inhibítorom ZVAD-fmk. Induktor apoptózy staurosporín bol použitý ako pozitívna kontrola.

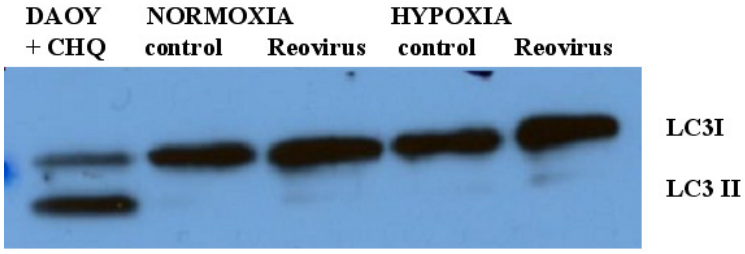
Na potvrdenie našich meraní o aktivovaných kaspázach, sme urobili kaspázovú esej. Zistili sme, že hladina indukovanej kaspázy-3 sa signifikantne nelíšila v jendotlivých hladinách kyslíka s výnimkou pôsobenia reovírusu v normoxii po dobu 48 hodín, kde se aktivita kaspázy 3 zvýšila viď Graf č.9



Graf č.9 Výsledky kaspázovej eseje s Daoy bunkami po infekcii reovírusom v normoxii a hypoxii. Cisplatina bola použitá ako pozitívna kontrola.

Aby sme zistili akými rôznymi mechanizmami reovírus vyvoláva typ bunkovej smrti v hypoxii i normoxii, zmerali sme western blotom hladiny premeny proteínu LC-3 z LC-3 I na LC-3 II, čo semikvantitatívne reprezentuje hladinu autofágie. Hladina štiepeného LC-3 II bola rovnaká nielen v hypoxických a normoxických vzorkách, ale i vo vzorkách infikovaných a neinfikovaných vírusom viď obrázok . Autofágia teda nie je typ smrti, ktorý reovírus vyvoláva v hypoxii., obrázok č. 6.

V našich pokusoch sme teda prvýkrát ukázali, že typ bunkovej smrti vyvolanej reovírusom nastáva mechanizmom nezávislom na kaspázach. A taktiež, bunková smrť indukovaná reovírusom nie je spôsobená autofágiou.



Obrázok č.7. Dôkaz autofágie konverziou LC3I na LC3II. Bunky Daoy boli kultivované v normoxii i v hypoxii s reovírusom i bez neho. Ako pozitívna kontrola boli použité bunky inkubované s chloroquínom.

Na hypoxiu je nahliadané ako na negatívny prognostický faktor malígnych nádorov, pretože okrem iných vplyvov spôsobuje aj rezistenciu voči rádio- i chemoterapii (Shannon A.M. et al., 2003, Um J.H. et al., 2004). Preto sú agens, ktoré pôsobia v normoxii ako i v hypoxii intezívne hľadané. Hypoxia indukuje rezistenciu oboma, na HIF-1 závislými a HIF-1 nezávislými mechanizmami (Rohwer N. a Cramer T., 2011). Aktuálny podiel rôznych transkripčných faktorov na hypoxiou-indukovanú rezistenciu voči apoptóze závisí na viacerých faktoroch (napr. typ buniek, stupeň a dĺžka hypoxie, typ proapoptotického stimulu). Na našom pracovisku sme ukázali dôležitú úlohu HIF-1α v neuroblastómových bunkových líniách v experimentoch s inhibíciou HIF-1α (H.Marikova: Significance of HIF-1α expression in neuroblastoma cell lines. Diploma thesis. Institute of chemical technology, Prague, 2011). V aktuálnej práci sme ukázali, že reovírus dokáže oboje, replikovať sa v hypoxickom nádorovom mikroprostredí a taktiež spôsobiť cytopatický efekt s následne vyvolanou bunkovou smrťou. Zistili sme, že veľká časť buniek v hypoxii podlieha smrti na kaspázach nezávislými mechanizmami. Úloha HIF-1α ako anti- alebo proapoptotického transkripčného faktoru je stále diskutovaná (Piret J.P. et al., 2002) a bolo publikované, že konštitutívna expresia HIF-1α obmedzuje replikáciu reovírusu v bunkách karcinómu obličiek a kolorekta (Cho I.R. et al., 2010). My sme však nezaznamenali zníženú hladinu HIF-1α spôsobenú prítomnosťou reovírusu v bunkových líniách odvodených z nádorov mozgu ako bolo popísané v karcinómových bunkách spomínaných vyššie (Cho I.R. et al., 2010). Možné vysvetlenie tohoto rozdielu spočíva zrejme v rozdielnej biológii skúmaných buniek alebo v použití iného percenta hypoxie v nastavenom systéme, Cho et al. používali 2% O_2 zatiaľčo my obvyklejšie 1%.

Preklinické štúdie potvrdili, že reovírus sa množí jedine v bunkách s aktivovaným génom rodiny *Ras* alebo jeho dráhy, čo sa nachádza v 60-80% ľudských malignancií. Oncolytics Biotech Inc. momentálne vedie klinické štúdie s reovírusom pod komerčným názvom Reolysin®. Dokončené štúdie, ktoré zahŕňali stovky pacientov, ukázali, že intratumorálne, vrátane intrakraniálneho a intravenózneho podania Reolysin®-u, je pacientami veľmi dobre znášané, tak ako spomínam v samostatnej časti v práci vyššie. Takisto bola testovaná kombinácia Reolysin®-u s chemo a/alebo rádioterapiou, pozri priloženú literatúru a tabuľku č. 2. Všetky tieto štúdie potvrdili potenciálny účinok reovírusu ako protinádorového liečiva. My sme ukázali jeho aditívnu výhodu, a to jeho schonosť množiť sa v hypoxickom nádorovom mikroprostredí. Jeho schopnosť množiť sa a spôsobovať cytopatický efekt v hypoxii nemizne, naopak, napriek pan-kaspázovému bloku je stále schopný navodiť apoptózu. Z toho vyplýva, že reovírus je schopný indukovať bunkovú smrť i nezávisle na kaspázach. Ukázali sme, že to nie je autofágia, čo reovírus vyvoláva v hypoxii. Napriek tomu, že stále nevieme presne, aký typ bunkovej smrti nastáva, táto informácia je dôležitým nástrojom v ďalšej práci s reovírusom v liečbe hypoxických neoplázií. Ak sa nám podarí podporiť jeho schopnosť indukovať bunkovú smrť v hypoxických podmienkach špecifickým spôsobom, máme nový, účinný protinádorový postup. Výsledky tejto práce sme spísali do publikácie, ktorá je momentálne posudzovaná pre publikáciu v *Journal of applied biomedicine.*

5. Priložené publikácie

Reovirus – possible therapy of cancer*
Minireview

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Oncolytic viruses infect, replicate in, and eventually lyse tumor cells but spare normal ones. In addition to direct lysis, a result of viral replicative cycle, viruses also mediate tumor cell destruction by inducing nonspecific and specific antitumor immunity. Some viruses express proteins that are cytotoxic to tumor cells. Viruses recognized as oncolytic agents can therefore be divided into three categories: 1/ naturally occurring viruses (e.g. Newcastle disease virus, vesicular stomatitis virus, autonomous parvoviruses, some measles virus strains, reovirus) that selectively replicate in tumor cells, in some instances owing to their relative resistance to interferon action; 2/ virus mutants in which some genes essential for replication in normal cells but evitable in cancer cells have been deleted (e.g.adenovirus ONYX 015 that replicates only in cells with defected p53 or herpes virus G207 which exacts the presence of ribonucleotide reductase); 3/ virus mutants modified by the introduction of tissue-specific transcriptional elements that drive viral genes (e.g.adenovirus CV706 that has PSA restricted expression E1A and E1B and adenovirus adMycTK that binds selectively on myc protein).

Reovirus is prevalent in the human population but not associated with any known human disease. Studies have shown that Reovirus multiplicate preferentially in tumor cells with activated gene of *ras* family or *ras-signaling pathway* while sparing normal cells. Activated *ras* or its pathway could be found in as many as 60–80% of human malignancies. In our studies we used cell lines that demonstrably express activated *ras*. We showed the cytopathic effect of reovirus (serotype 3 strain Dearing) on medulloblastoma cell lines and compared it with its acting on normal human fibroblasts. Oncolytics Biotech Inc. is currently guiding three Phase I or Phase I/II Reolysin studies, and has completed two clinical studies and concluded enrolment in a third one.

Key words: oncolytic viruses, reovirus, clinical trials

Malignant tumors remain one of the main causes of death in all developed countries and their incidence is still rising. Fortunately, progress has been made in the overall survival of cancer patients after introduction of improved imaging and diagnostic techniques; elucidation of the molecular processes that cause cancer, and further comprehension of treatment using combined chemo- and radiotherapy. However, survival has not improved substantially with current chemotherapy and radiotherapy in patients diagnosed with metastatic disease and certain high-incidence tumors such as brain tumors, pancreatic, colorectal, and liver carcinomas. Surgery and radiation therapy afford only local control, therefore are not effective in metastatic diseases and chemotherapy is limited by toxicity and by primary or secondary chemoresistance to the drugs in use. This incepts usually due to tumor cells developing different mechanisms that override cell death caused by chemotherapy and radiotherapy. As a result, resistance to treatment through clonal expansion of genetically resistant tumor cells occurs. Much effort has been directed toward finding alternate pathways that would complement therapeutic induction of apoptosis, overcome multidrug resistance, and ultimately improve overall cure rates. In view of this, several new classes of anticancer agents are being promoted as potential supplements to current anticancer therapy. They include monoclonal antibodies, biological response modifiers, angiogenesis inhibitors, modulators of signal transduction, gene therapy including antisense oligonucletides, telomerase and kinase inhibitors. An additional group of agents includes viruses that infect, replicate in, and eventu-

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ally lyse tumor cells but spare normal ones. The possibility of using viruses as oncolytic agents was originally recognized in cases of unintentional exposure. The virus-induced remissions occurring either naturally [1] or induced by vaccination [2] stimulated research on the oncolytic activity of a variety of viruses.

Oncolytic viruses

Revolutionary advances in molecular biology and genetics have led to a fundamental understanding of the replication and pathogenicity of viruses and the carcinogenesis. These advances have allowed novel agents to be engineered to enhance the antitumoral potency as well as safety of oncolytic viruses. Oncolytic viruses were evolved to infect cells, replicate inside the host, induce cell death, release the viral particles, and finally to spread in human tissues. Replicating viruses "self-amplify" that potentially leads to maximized dosing at the desired site of action, while a lack of replication in normal tissues can result in efficient clearance and reduced toxicity. Selective replication within tumor tissue can theoretically increase the therapeutic index of these agents enormously. Furthermore, oncolytic viruses can mediate the destruction of tumor cells by several mechanisms. In addition to direct lysis, a result of viral replicative cycle, viruses also mediate tumor cell destruction by inducing nonspecific and specific antitumor immunity. Some viruses express proteins that are cytotoxic to tumor cells (adenoviruses express cytotoxic proteins E3 and E4ORF4) [3]. Viral infection of cells elicits an immune response that consists of cytokine generation (interferons α, β and γ, TNFα, and several interleukins) and infiltration of macrophages, neutrophils, and NK cells. Therefore, since activation of classical apoptotic pathways in the cancer cell is not the exclusive mode of killing, cross-resistance with standard chemotherapeutics or radiotherapy is much less likely to occur. On the other hand, the effect of immune response is also likely to destroy replicating virions and so limit the direct lytic effect [4]. TODA et al [5] showed that treatment of tumors in mice with genetically modified oncolytic herpes virus G207 also elicited systemic immunity against other tumors in which virus was not detected through a cytotoxic T cell response. Immunosuppresion by corticosteroids decreased efficiency of G207 in transplanted human tumor [6]. On the other hand HIRASAWA et al [7] found increased efficiency of reovirus in mice tumor after co-administration of cyclosporine A and anti CD4 and anti CD8 antibodies. It remains to be determined which mechanisms are involved in antiviral immunity and which in anticancer immunity.

As with any anticancer therapy, the cytotoxic effects of the treatment upon the normal tissue surrounding the tumor should be minimized. The ideal oncolytic virus would express such high specificity for tumor cells even when delivered systemically; it would localize to act directly on cancer cells. Additionally, the virus would replicate quickly in dividing as well as quiescent cancer cells to high titers. Further would disseminate throughout the tumor mass, destroying cells directly or sensitizing them to the action of other therapeutic agents, but would still remain non-dangerous to surrounding normal tissue. The ideal virus must also be able to replicate efficiently in the context of a developing, or even a pre-existing antiviral immune response. This may require expression of viral proteins that are involved in suppression of the antiviral immune response. Virus would therefore cause minimal immunological reaction, and would be well tolerated by patients. Furthermore, infection with the virus should stimulate an effective antitumor immune response that would lead to the destruction of metastases [8]. Much work over the last three decades has been performed with the aim of producing such an ideal virus.

Oncolytic viruses, which have been tested as cancer therapeutics, have either been naturally selected or have been genetically engineered to grow specifically in and kill tumor cells. Viruses recognized as oncolytic agents can therefore be divided into three categories: 1/ naturally occurring viruses (such as Newcastle disease virus, vesicular stomatitis virus, autonomous parvoviruses, some measles virus strains, reovirus [9]) that selectively replicate in tumor cells, in some instances owing to their relative resistance to interferon action [8]; 2/ virus mutants in which some genes essential for replication in normal cells but evitable in cancer cells have been deleted (e.g.adenovirus ONYX 015 that replicates only in cells with defected p53 or herpes virus G207 which exacts the presence of ribonucleotide reductase) [4]; 3/ virus mutants modified by the introduction of tissue-specific transcriptional elements that drive viral genes (e.g. adenovirus CV706 that has PSA restricted expression E1A and E1B and adenovirus adMycTK that binds selectively on myc protein) [10]. Each of these agents has shown tumor selectivity *in vitro* and/or *in vivo*, with many of these agents following intratumoral, intraperitoneal and/or intravenous routes of administration. Overview of the most crucial oncololytic viruses shows Table 1.

There is now clear evidence in pre-clinical models that oncolytic viruses have great potential to become important new therapeutics. Results from Phase I and II intratumoral trials are beginning to supervene and it seems that the current oncolytic viruses are safe and have reduced acute side effects when compared with many other conventional cancer therapeutics [11, 12]. The first virus studied in clinical trials is the adenovirus ONYX-015, which has been the subject of 18 phase I and II clinical trials with published results, starting in 1996. To date, more than 250 and 170 patients have been treated with ONYX-015 and Newcastle disease virus respectively [13]. Indeed evidence from both pre-clinical and clinical studies suggests that combining replication-competent viruses with standard anticancer treatments such as chemotherapy and radiotherapy may result in greater therapeutic benefit [14–17]. ONYX-015 became the first virus combined with chemotherapy to undergo clinical trials [18].

Table 1. Overview of oncolytic viruses in clinical trials (modified according Kirn DH Replication-selective microbiological agents: fighting cancer with targeted germ warfare. J Clin Invest 2000; 105: 837–839)

Virus family	Oncolytic virus	Specificity	Genetic alterations
Adenovirus	ONYX-015	cells lacing p53 function	E1B-55kD, E3b deletion
	CV 706	prostate cells	E1A expression driven by PSA element, deletion E3
	CV 787	prostate cells	E1B expression driven by PSA element
	Ad5-CD/tk-rep	cells lacing p53 function	E1B-55kD deletion
	adMycTK	myc expressing cells	Myc-Max binding motif
Herpes simplex	G207	proliferating cells	ribonucleotide reductase disruption and deletion of gamma 34.5
	NV1020	proliferating cells	deletion of gamma 34.5
	1716	proliferating cells	deletion of gamma 34.5
Vaccinia	wild type +/– GM-CSF	unknown	wild type
Newcastle disease v.	73-T, PV 701, Ulster strain, MTH-68/N	unknown	wild type
Autonomous parvoviruses	H-1	transformed cells-↑proliferation, ↓differentiation, ras, p53 mutation	wild type
Reovirus	Reolysin	ras-pathway activation	wild type

Table 2. Clinical trials with Reovirus (modified according www.oncolyticsbiotech.com)

Clinical Study/Trial	Application	Objective	Results	Cancer type	Therapy
Phase I Study	intratumoral	safety, maximum tolerated dose	no serious adverse events related to the virus	progressing solid tumors	momotherapy
T2 Prostate Cancer Trial Phase I	intratumoral	safety, histopathology	evidence of apoptosis tumor cell in 4 of 6 patients, no safety concerns	prostatic cancer	monotherapy
Phase I/II Recurrent Malignant Glioma Trial	intratumoral	safety	well tolerated	recurrent malignant glioma	monotherapy
		safety	not finished		with chemotherapeutics and radiation therapy
Phase I Systemic Administration Trial	intravenous	safety	not finished	advanced primary or metastatic solid tumors	monotherapy
		tumor and immune response	not finished		
Phase I Combination Reolysin/Radiation Therapy Trial	intratumoral	feasibility, safety and anti-tumor effects	not finished	advanced cancer	with radiation
		evidence of any anti-tumor activity			
Phase I Systemic Delivery Trial	intravenous	maximum tolerated dose, dose limiting toxicity,	not finished	advanced or metastatic tumors	monotherapy
		viral replication, immune response, any evidence of antitumor activity	not finished		
Phase I/II Recurrent Malignant Gliomas Trial	infusion	maximum tolerated dose, dose limiting toxicity, safety	not finished	malignant gliomas	monotherapy
		viral replication, immune response, antitumor activity	not finished		

In some cases virus therapy in combination with chemotherapeutics has provided enough evidence of efficacy to warrant proceeding to phase III trials [18–21]. The majority of clinical studies to date involve intratumoral treatments. Systemic treatment of cancer using oncolytic viruses is clearly the next key step for broader applicability. Recently, intravenous treatment of advanced cancer patients using oncolytic viruses has included results of studies with PV701 for maximum tolerated dose determination [23] and of trial with ONYX-015 [21]. Adenovirus ONYX-015 was also administered by hepatic artery infusion in patients with gastrointestinal carcinoma metastatic dissemination to the liver [22]. However more studies with humans need to be initiated or more fully developed for both locoregional and systemic treatment approaches. We need to know how reliable preclinical models predict outcomes in humans. It is likely that questions concerning viremia, virus clearance, humoral and cellular immune responses, tumor to tumor spread, and virus stability can only be answered by testing in humans. Fine tuning and optimization of viral therapeutics will best be done in a Phase I setting.

Knowledge of mechanisms affecting efficiency of oncolytic viruses and of potentiation of their efficiency by cytostatics and/or radiotherapy is important for their use in therapeutic protocols. Therefore we started preclinical experiments with reovirus. We intend to study immunological

mechanisms which may potentiate its efficiency; however, antibodies may neutralize the virus.

Reovirus

Reovirus (an acronym for *r*espiratory *e*nteric *o*rphan) is highly prevalent in the human population but not associated with any known human disease [24]. It has been isolated from the respiratory and gastrointestinal tract and is considered an orphan virus, because it lacks clinical symptoms [25]. It is found naturally in sewage and water supplies. By the age of 12 years, half of all children show evidence of reovirus exposure and by adulthood, most people have been exposed. As mentioned above, reovirus is non-pathogenic, meaning there are typically no symptoms from infections. The link to its cancer-killing ability was established after the reovirus was discovered to reproduce well in various cancer cell lines. Serotype 3 Dearing strain is under clinical investigation in its natural, non-mutated form.

Taxonomically, it is a member of the *Reoviridae* family. These are non-enveloped viruses with icosahedron shape and size ranging from 70 to 85 nm. In addition to the inner core (size 60–70 nm), they posses an outer capsid structure. Their genome is segmented and contains 10–12 pieces of double-stranded RNA and its size is 24 kb. Reovirus represents one of the *Reoviridae* genera that infect human beings [24].

The reovirus lytic cycle begins with attachment of a virion to sialic acid residues on the cell surface via the trimeric σ1 cell attachment protein, which protrudes from the 12 vertices of the icosahedral capsid [26]. Following attachment, clathrin-coated pits form and the virus enters by receptor-mediated endocytosis. Within the resulting endosome/lysosomes, acid-dependent proteolysis of viral outer capsid proteins σ3 and μ1/μ1c begins, generating an intermediate subviral particle (ISVP). Later on, degradation of 3 occurs, which theoretically exposes μ1/μ1c, allowing for penetration of the ISVP across the lysosomal membrane. μ1/μ1c has been shown to be capable of disrupting membrane bilayers *in vitro* [27]. μ1/μ1c is also myristoylated which may aid in ISVP/membrane fusion [28]. Following this step, primary transcription of 10 capped, full-length transcripts takes place, mediated by the viruses' double-stranded RNA-dependent RNA polymerase. Primary transcripts are translated using host machinery and subsequently associate with primary translation products to form RNA assortment complexes.

Final synthesis of minus strand genomic RNA occurs within these nascent particles and secondary transcription of late viral mRNAs begins. The synthesis of viral mRNA within the virus particle is the characteristic feature of reoviruses' replication. Late viral protein synthesis from secondary transcripts often coincides with a decrease in host protein synthesis [29]. Final assembly of the outer capsid yields progeny reovirus particles, leading to cell lysis and death. In the infected culture the maximal virus yield is achieved 15–18 hrs post infection, with 200–2000 plaque forming units per cell. Quite typical for the harvested virus population is a high ratio between physical and infectious particles (1:100 to 1:1000). This occurs most probably due to the predominant presence of incomplete, defective particles arising in the course of virus replication. Reoviruses are stable over a long period of time and are resistant to exposure to high ionic strength, relatively high temperature (exceeding 50 °C) and extreme pH values.

Recent studies have shown that Reovirus propagates preferentially in tumor cells with activated gene of *ras* family or *ras-signaling pathway* while sparing normal cells [30]. Activated *ras* or its pathway could be found in as many as 60–80% of human malignancies [31]. Studies have shown that reovirus fails to productively infect NIH-3T3 cells unless they express activated *ras* [31, 32]. The reason why cells with activated *ras* pathway can be productively infected by reovirus is associated with the disruption of the cell defense against viral infection. In non-transformed, reovirus infected cells, after primary transcription, the double-stranded RNA-dependent protein kinase (denoted PKR) is activated. By phosphorylating the initiation factor eIF2-α, PKR shuts off viral protein synthesis. This phosphorylation is inhibited when the *ras* signaling pathway is activated, resulting in viral translation and subsequent entrance into the viral lytic cycle. In tumor cells with an activated *ras* pathway, reovirus is able to freely replicate and eventually kill host tumor cells. As cell death occurs, progeny virus particles are free to infect surrounding cancer cells. This cycle of infection, replication and cell death is believed to be repeated until there are no longer any tumor cells carrying an activated *ras* pathway available. The activation of the *ras* pathway can be mimicked in normal cells by treating these cells with 2-aminopurine (2-AP) which prevents the activation of PKR [32].

In our studies we used cell lines that demonstrably express activated *ras*. We showed the cytopathic effect of reovirus (serotype 3 strain Dearing) on medulloblastoma derived cell lines and compared it with its acting on normal human fibroblasts that are believed to have their *ras cascade* inactivated. The cytopathic effect on medulloblastoma occurred within four days, while fibroblasts remained untouched (Fig. 1). Reovirus significantly potentiates effect of cisplatin on medulloblastoma and glioblastoma derived cell lines [33]. Reovirus we used is identical with REOLYSIN® produced by Oncolytics Biotech Inc.

This company is currently guiding three Phase I or Phase I/II Reolysin studies in the United Kingdom and the United States, and has completed two clinical studies and concluded enrolment in a third study in Canada. The recent clinical program for Reolysin addresses various human cancers and uses various modes of administration including local delivery, systemic delivery and delivery in combination with radiation therapy. Phase I/II recurrent malignant glioma study in the United States is in current state of preparation (www.oncolyticsbiotech.com).

It has been the failure of conventional anticancer treatment

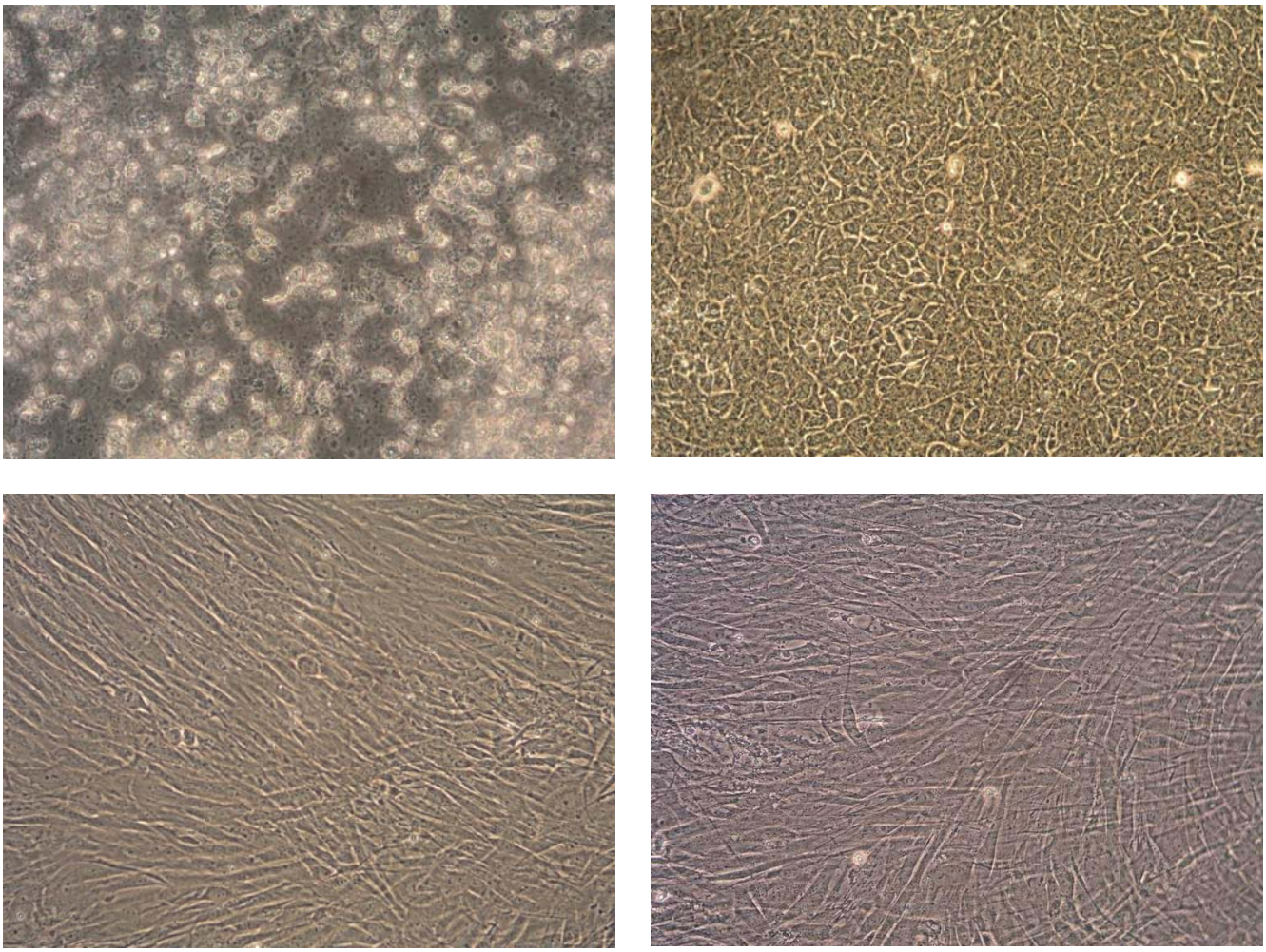

Figure 1. Effect of reovirus on medulloblastoma derived cell line ATCC HTB 186 (Daoy) (right) 96 hours after infection and on normal human fibroblasts (left). Control without virus (bottom) medulloblastoma derived cell line ATCC HTB 186 (Daoy) (right) and normal human fibroblasts (left). Magnitude 40x.

that has inspired researchers all over the world to look for new drugs which could efficiently kill even the chemoresistant tumor cells. From all different groups of current agents discovered, replication competent viruses seem promising for cancer treatment mainly because of their ability to amplify themselves and spread throughout the tumor mass. Additionally, they can possibly express foreign proteins that fortify their own innate cytolytic potential. Significant progress has been made in targeting viruses to particular cell types, but a real tumor-specific virus is yet to be constructed. However it still seems a little ironic, that viruses might be used to combat neoplasms, since approximately 15% of the incidence of human cancer is attributable to virus infection [34]. It is probable that in the future an extent group of viruses that are able to target different cells will suit for use as anticancer agents. As many viruses lyse the cells in which they replicate, the suggestion that viruses might potentially be used to destroy specific cell populations is not altogether surprising.

Conclusion

Oncolytic viruses represent a rapidly expanding novel therapeutic platform for cancer. Hundreds of viruses are now being tested preclinically, and approval has been sought and/or testing in humans has been initiated in at least ten ones. Only a few therapeutic areas within biotechnology have ever expanded so quickly.

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Reolysin®

Oncolytic Virus

Human reovirus-based cancer therapy

EN: 287727

Abstract

Oncolytic viruses infect, replicate in and lyse tumor cells while sparing normal cells. In addition to direct lysis, viruses induce antitumor immunity and some viruses express cytotoxic proteins. Oncolytic viruses can be divided into three categories: 1) naturally occurring viruses that selectively replicate in tumor cells; 2) virus mutants in which some genes essential for replication in normal cells but unnecessary in cancer cells have been deleted; and 3) virus mutants modified by the introduction of tissue-specific transcriptional elements that drive viral genes. Preclinical studies have shown that reovirus proliferates only in tumor cells with activated genes of the *RAS* family or its pathway. Activated *RAS* or its pathway can be found in 60-80% of human malignancies. Oncolytics Biotech is currently conducting clinical studies with the human reovirus-based cancer therapy Reolysin®. Four completed studies in cancer patients demonstrated that intratumoral (including intracranial and intravenous) application of Reolysin® is well tolerated.

Background

Despite the advances in cancer screening, diagnostics and treatment that have decreased mortality in patients, cancer remains one of the main causes of death in all developed countries, and its incidence is on the rise. Current efforts to improve commonly applied cancer therapies are aimed at enhancing drug efficacy while maintaining acceptable levels of toxicity. In order to succeed, innovative therapeutics have been designed to target tumor-specific attributes to permit higher doses with less side effects. One such approach utilizes oncolytic viruses (OVs) that infect, replicate in and lyse tumor cells while sparing normal cells. The possibility of using viruses as oncolytic agents was originally recognized in cases of unintentional exposure. Virus-induced remissions occurring either naturally (1) or induced by vaccination (2) stimulated research on the oncolytic activity of a variety of viruses.

OVs were developed to infect tumor cells, reproduce inside their host, induce cell death, release offspring viral particles and spread across human tumors. Replication-competent viruses “self-amplify”, which potentially leads to maximized dose at the desired site of action, while the absence of replication in normal tissues can result in reduced side effects. Selective replication within tumor tissue can increase their therapeutic index. However, physical barriers such as necrotic areas, stromal cells, extracellular matrix or basal membrane may further limit the distribution of the virus. The significance of diffused virus application was clearly demonstrated in mathematical models of viral replication (3).

The ideal OV should be confined exclusively and specifically to tumor cells, even when delivered systemically, in order to act directly on target cancer cells. The ideal virus should replicate quickly in both dividing and quiescent cancer cells. Furthermore, it should disseminate throughout the tumor mass, destroying its host, and at the same time not be harmful to normal tissues. The perfect virus must also be able to replicate efficiently in the context of innate antiviral immune responses. This may require expression of viral proteins that are involved in the suppression of antiviral immunity. The virus should cause a minimal immune reaction and should be well tolerated by patients. Furthermore, infection with the virus should stimulate an effective antitumor immune response that would lead to the destruction of metastases (4).

Much work over the last three decades has been performed with the aim of producing such a virus. OVs that have been tested as anticancer drugs have either been naturally selected or have been genetically engineered to grow specifically in and kill cancer cells. OVs can be divided into three categories: 1) naturally occurring viruses (*e.g.*, Newcastle disease virus, vesicular stomatitis virus, autonomous parvoviruses, some measles virus strains, reovirus [5]) that selectively replicate in tumor cells; 2) virus mutants in which some genes essential for

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replication in normal cells but unnecessary in cancer cells have been deleted (*e.g.*, the adenovirus ONYX-015, which replicates only in cells with mutant *p53*, or the herpesvirus G207, which requires the presence of ribonucleotide reductase [*ICP* gene]) (6); and 3) virus mutants with tissue-specific transcriptional elements that drive viral genes (*e.g.*, the adenovirus CV706, which has prostate-specific antigen [PSA]-restricted expression of *E1A* and *E1B*, and the adenovirus adMycTK, which selectively binds myc protein) (7). All of these viruses are tumor-selective *in vitro* and/or *in vivo*. Many of these agents have already been clinically tested using intratumoral, intraperitoneal (i.p.) and/or intravenous (i.v.) routes of administration.

OVs can mediate the destruction of tumor cells by mechanisms other than direct lysis. OVs may effectively induce adaptive antitumor immunity that comprises both antibody and T cell responses targeting tumor-associated antigens (TAAs) (8-10). Some viruses express proteins that are cytotoxic to cancer cells (*e.g.*, adenoviruses express cytotoxic proteins E3 and E4ORF4) (10), and importantly, Schulz *et al.* showed that cells infected with virusues were more effective at delivering nonviral antigens for cross-priming of antigen-presenting cells (APCs) *in vivo* (11).

Viral infection of cells induces an immune response that consists of cytokine production (interferons α, β and γ, tumor necrosis factor α [TNF-α] and several interleukins) and infiltration of cytotoxic cells, such as macrophages, neutrophils and natural killer (NK) cells. Since activation of apoptotic pathways in cancer cells is not the main mode of the destruction induced by OVs, cross-resistance with standard chemotherapeutics or radiotherapy is not frequent. However, an immune response is also likely to destroy replicating virions, thus limiting their effects (5). Although neutralizing antibodies and the complement system do not limit therapeutic efficacy in the case of intratumoral injection of OVs, they may significantly restrict systemic therapy (6). Immunosuppression by corticosteroids decreased the efficiency of G207 (double mutant of the herpes simplex virus type 1 [HSV-1] with deletions at both γ 34.5 (RL1) loci and a *lacZ* gene insertion inactivating the *ICP6* gene) in transplanted human tumors (8). On the other hand, Hirasawa *et al.* (9) found increased efficiency of a reovirus in mouse tumors after the application of ciclosporin and anti-CD4 and anti-CD8 antibodies. It remains to be determined which mechanisms are involved in antiviral immunity and which contribute to the anticancer effect.

Reovirus (an acronym for Respiratory Enteric Orphan viruses) is one of the replication-competent, naturally occurring viruses that preferentially kill tumor cells (12). Research into the mechanism of the tumor cell selectivity of reovirus revealed that it replicates favorably in the presence of activated Ras signaling, which is common in cancer cells (13).

Reovirus replicates in the cytoplasm and comprises two concentric icosahedral protein capsids with trimeric σ1 proteins that protrude from vertices. The capsid surrounds the genome consisting of 10 segments (large L1, L2 and L3, medium M1, M2 and M3, and small S1, S2, S3 and S4) of double-stranded (ds) RNA (14). Each dsRNA segment encodes a single protein, except for the S1 gene segment, which is bicistronic. Proteins are denoted λ, μ and σ according to the RNA segment from which they are transcribed. Reovirus encodes its own polymerases essential for replication of the viral genome and therefore is not dependent on the S phase of the host as some DNA viruses are (4).

Reoviruses are ubiquitous viruses that have been isolated from a wide variety of mammalian species, including humans. In humans, reoviruses are commonly isolated from the respiratory and gastrointestinal tract, but they are not associated with any known disease and are therefore considered to be nonpathogenic (15). Thus, they were classified as orphan viruses (a virus which is not associated with any known disease). According to their hemagglutination activity, three serotypes of reovirus have been described. Laboratory strains of each serotype were isolated and designated serotype 1 Lang, serotype 2 Jones, serotype 3 Abney and serotype 3 Dearing (T3D). All three serotypes of reovirus are found ubiquitously in the environment, particularly in still water or sewage water. As many as 50% of adults aged 20-30 years have already been exposed to reovirus and carry antibodies against the virus, and seropositivity has been detected in 70-100% of older individuals (16, 17).

The lytic cycle of reovirus is complex and consists of many steps. It begins with the attachment of the virion to the receptor of the host cell via σ1 protein and the virus enters the cell by receptor-mediated endocytosis. The σ1 protein is a fibrous trimer composed of an elongated tail domain inserted into the virion and a globular head domain that sticks out from the virion surface (18). The head and tail regions of T3D σ1 contain receptor-binding domains. The domain in the tail binds α-linked sialic acid (19, 20), whereas the domain in the head binds junctional adhesion molecule 1 (JAM-1) (21). σ1 protein of T3D can be dissociated by intestinal proteases such as trypsin or chymotrypsin (22). Reovirus attaches to cells via an adhesion-strengthening mechanism by which initial low-affinity binding to sialic acid facilitates secondary higher affinity binding to JAM-1. The capacity of T3D reovirus to bind sialic acid influences infection of cultured cells (23). However, although ligation of sialic acid and JAM-1 is necessary for reovirus-induced cell death, viral attachment to the cell surface alone is not enough. Inhibitors of acid-dependent viral disassembly block apoptosis activated by reovirus, which indicates the requirement for post-attachment entry steps (24). Within the endosome, proteolysis of viral outer capsid proteins gives rise to an intermediate subviral particle (ISVP). Receptor binding and disassembly must occur within the same cellular compartment to elicit an apoptotic response. A critical component of the signaling cascade that leads to apoptosis of reovirus-infected cells is the transcription factor NF-κB (25). Reovirus also activates c-Jun *N*-terminal kinase (JNK) and extracellular signal-regulated kinase

(ERK) (26), but the involvement of these signaling molecules in NF-κB activation and apoptosis induction is not understood. Triggered ISVPs penetrate through the endosomal membrane. Subsequently, the transcription of 10 RNA segments mediated by viral dsRNA-dependent RNA polymerase proceeds. Later, the synthesis of RNA minus-strand occurs and secondary transcription of late viral mRNAs begins. Final composition of the outer capsid yields viral particles that induce cell lysis (27). Moreover, viral transcription is not indispensable, as inhibitors of viral RNA synthesis do not diminish the capacity of reovirus to induce apoptosis (24, 28).

Reovirus does not replicate in normal mouse fibroblasts, but fibroblasts transfected with activated Ras, epidermal growth factor receptor (EGFR, ErbB-1) or V-erbB are lysed by uninhibited reovirus replication (12). The mechanism of preferential reoviral tropism in *RAS*-transformed cells has not been fully defined, but a defective cellular antiviral response triggered in these cells is obvious. In normal cells, reoviral dsRNAs activate PKR (protein kinase RNA-activated), which in turn phosphorylates the α subunit of initiation factor 2 (eIF-2-α). This phosphorylation shuts off any further protein translation and thus inhibits the initiation of translation of viral transcripts. In contrast, in cells with constitutive Ras activation, the phosphorylation of eIF-2-α is inhibited, resulting in viral translation and subsequent entry into the viral lytic cycle (13). Furthermore, recent evidence suggests that Ras transformation enhances viral uncoating, infectivity and virion release (29), and makes cells more sensitive to virus-induced apoptosis (30). Since activating mutations of the proto-oncogene *RAS* occur in about 60-80% of all human tumors, *e.g.*, pancreatic (90%), sporadic colorectal (50%) and lung (40%) carcinomas and myeloid leukemia (30%) (31), reovirus appears to be a good tool for inhibiting such cell populations.

Generally speaking, reovirus induces cell cycle arrest at G1 and G2/M and apoptosis, and selectively activates mitogen-activated protein kinase (MAPK) cascades. Reovirus-induced apoptosis involves members of the TNF-related apoptosis-inducing ligand (TRAIL) family and is associated with the activation of both death receptor- and mitochondrial-associated caspases (32). However, it is still not known what gene product(s) of reovirus are responsible for these properties.

In addition, reovirus may also activate the host immune system to enhance antitumor activity. Errington *et al*. showed that reovirus induced phenotypic dendritic cell (DC) maturation and the production of inflammatory cytokines, and that infected DCs could in turn elicit NK and T cell-mediated innate antitumor activity (33). In other experiments, they showed that the inflammatory response generated by reovirus-infected melanoma cells caused bystander toxicity against reovirus-resistant tumor cells and activated human myeloid dendritic cells *in vitro* (34).

Since reovirus, a dsRNA virus, is an efficient inducer of interferon α and β, it is believed that a host interferon response may play an important role in oncolysis (35). On the contrary, resistance to reovirus was demonstrated. In an *in vitro* study using a human fibrosarcoma-derived cell line that carried *RAS* mutation resistance to reovirus associated with persistent reovirus infection, elevated PKR phosphorylation and decreased cathepsin B activity were documented (36).

Preclinical Pharmacology

In an *in vitro* study, all primary glioma cultures from patients and 20 of 24 established glioma cell lines treated with reovirus were destroyed, whereas all meningioma primary cultures were spared. In *in vivo* experiments, reovirus therapy prolonged survival in two orthotopic intracerebral mouse glioma models and caused significant cytoreduction and tumor regression in two subcutaneous immunodeficient mouse models of malignant glioma (37, 38). Direct intracerebral injection of reovirus appeared to be safe in mice, rats and cynomolgus monkeys. Survival of nude mice with medulloblastoma cell lines implanted orthotopically was prolonged after intratumoral injection of live reovirus compared to injection of an inactivated reovirus (39). In an orthotopic medulloblastoma model in nude mice, multiple intratumoral reovirus injections were given. As expected, all control animals treated with inactivated virus developed spinal cord or leptomeningeal metastases, whereas none of the animals treated with live virus had detectable metastases (40). The investigators suggested that metastatic tumor cells were selected for high Ras activity and thus presented a favorable target for reovirus. This study also suggested that reovirus therapy might prevent both local invasion and metastatic tumor spread.

In another study, human breast cancer xenografts were inoculated in both the left and right hind flanks and reovirus was injected into one flank tumor only. Reovirus replication and tumor regression were observed at both injected and noninjected contralateral sites. In addition, reovirus could replicate not only in breast cancer cell lines, but also in surgical specimens from breast cancer patients (41). Furthermore, the ability of reovirus to treat breast tumors established in the brain was evaluated. Intracranial reovirus administration prolonged survival in nude mice, as did intrathecal reovirus administration in immunocompetent rat models. Both types of administration prevented local tumor invasion of breast cancer and metastases. Reovirus did not cause mortality when administered intracranially at doses up to 1×10^8 plaque-forming units (pfu) in *nu/nu* mice (42).

Reovirus also demonstrated activity against mouse Lewis lung cancer metastasis following i.v. administration, 65-80% of the tested mice showing regression of their tumors (43).

Reovirus was able to infect all five human pancreatic cancer cell lines tested *in vitro*. Elevated Ras activity was confirmed in these cell lines. Using two pancreatic cancer cell lines in a unilateral tumor xenograft model in nude mice, tumor growth was suppressed by intratumoral injection of reovirus. In addition, local injection of reovirus had

systemic antitumor effects in a bilateral xenograft model, with regression of both injected and uninjected xenografts being observed (44).

Reovirus exhibited significant antitumor activity against all four pediatric sarcoma (Ewing's sarcoma and rhabdomyosarcoma) xenografts tested (45). Reovirus also killed both human melanoma cell lines and freshly resected tumors, and intratumoral administration caused regression of melanoma in a xenograft model *in vivo* (34).

Reoviral irrigation, both immediate and delayed, of squamous cancer-contaminated wounds in SCID mice resulted in a significant reduction in tumor recurrence. Squamous cancer is susceptible to reovirus *in vitro*, and therefore this seems to be a suitable model of local adjuvant therapy after surgically induced complete remission (46).

Reovirus is not inactivated by clinically relevant doses of irradiation and radiation facilitates its cytotoxicity in tumor cell lines (head and neck, colorectal and breast cancer) *in vitro* and in syngeneic tumor xenografts (34, 47, 48). Clinical studies with reovirus in combination with radiotherapy have started based on these experiments. Treatment of human colon cancer cell lines with reovirus and gemcitabine resulted in *in vitro* and *in vivo* synergy (tumor was injected into the flanks of nude mice) (49). Intratumoral inoculation of reovirus in subcutaneous tumors induced in mice by human papillomavirus type 16 (HPV-16)-transformed TC-1 cells resulted in only a small decrease in tumor growth, but never in complete cure. When using cyclophosphamide in combination with viral treatment, a synergistic effect resulting in tumor suppression was observed. The best results were obtained when repeated cyclophosphamide administration was followed by reovirus treatment. A synergistic effect for reovirus and cyclophosphamide (coadministered with an S9 fraction, which is necessary for cyclophosphamide activation) was also found *in vitro* (manuscript submitted for publication). Similar effects of reovirus combined with cyclophosphamide were described in different *in vivo* models (50). Combined reovirus and radiotherapy led to statistically significant increases in cytotoxicity both *in vitro* and *in vivo*, particularly in those cell lines with moderate susceptibility to reovirus alone. The enhanced cytotoxicity of the combination occurred independently of treatment schedule (48).

Immunodeficient mice with tumors derived from the Burkitt's lymphoma Raji cell line were cured by i.v. reovirus, while inactivated reovirus was ineffective (51). Primary lymphoma samples collected from patients were tested *in vitro* and 100% of chronic B cell lymphoid leukemias and 100% of diffuse large B cell lymphomas were sensitive to reovirus, but the majority of follicular lymphomas were resistant (51, 52). Reovirus did not affect $CD34^+$ stem cells or their clonogenic potential. *Ex vivo* use of oncolytic virus seems to be promising for purging autologous hematopoietic stem cell harvests, as shown in *in vitro* studies with experimentally contaminated peripheral blood apheresis products (52). Mice with L1210 leukemia were treated with BCNU and subsequently with reovirus. Complete remission of tumor was observed in 80% of mice, and cured animals were resistant to challenge with L1210 leukemia, although they remained susceptible to challenge with heterologous tumor (53). One may speculate that reovirus potentiates the immune response to tumor antigens.

Clinical Studies

The positive preclinical findings led to the development of Reolysin® (reovirus serotype 3 strain Dearing), which is now being used in clinical trials as a powerful anticancer agent against tumors with an activated *RAS* oncogene or Ras pathway. Oncolytics Biotech has been issued patents that cover the pharmaceutical use of reovirus in the treatment of *RAS*-mediated cancers and the manufacture of reovirus in animal component-free media (54). A survey of clinical trials with Reolysin® is presented in Table I.

Intratumoral reovirus administration in a phase I study in patients with recurrent subcutaneous tumors demonstrated that dose escalation up to 10^{10} pfu did not cause any serious adverse events and no dose-limiting toxicities were found. The only adverse events were headache and transient flu-like symptoms. As a secondary endpoint in the study, tumor responses were also evaluated. Eleven of 18 patients (61%) demonstrated some response to viral therapy, with 32-100% tumor regression, and partial regression was documented in noninjected tumors in some patients (55).

Preliminary results of a clinical study showed that the combination of intratumoral Reolysin® administration and radiation was well tolerated. Three of 8 patients (esophageal, squamous skin and colorectal carcinoma) had significant partial responses. In addition, a patient with metastatic esophageal cancer also had tumor reduction in nonirradiated mediastinal disease (47, 56).

In another study, a total of 33 patients were treated i.v. with Reolysin®. PSA decreased by 50% in a patient with metastatic prostate cancer, with evidence of tumor necrosis on CT scan. Two patients with metastatic colorectal cancer had carcinoembryonic antigen (CEA) reductions of 60% and 27%. One patient with metastatic bladder cancer had a minor response. Reolysin® was well tolerated, with minimal toxicity, and the maximum tolerated dose was not reached (57, 58).

Twelve patients with recurrent malignant gliomas were treated with intratumoral Reolysin®. There were no grade 3 or 4 adverse events related to the administration of reovirus. One patient has stable disease and 11 patients had progressive disease (59, 60).

A prostate cancer trial was designed to evaluate the safety and efficacy of intratumoral administration of Reolysin® for the treatment of cancer restricted to stage T2. Patients received an intratumoral injection of Reolysin® and thereafter were monitored for 3 weeks, and at that time the prostate was removed. The primary efficacy endpoint was the response rate, as measured by pathological examination of the tumor. The pathological data showed evidence of apoptosis in 4 of 6 patients. Results of the

Table I: Clinical trials with Reolysin® (adapted from www.oncolyticsbiotech.com).

Cancer	Trial program	Phase	Status
Advanced pancreatic, lung, ovarian	I.v. administration with cyclophosphamide	I/II	Approved
Metastatic melanoma	I.v. administration as monotherapy	II	Protocol field
Ovarian	I.v. and i.p. administration	I/II	Pending
Sarcomas metastasized to lung	I.v. administration as monotherapy	II	Ongoing
Advanced pancreatic, lung, ovarian	I.v. administration with gemcitabine	I/II	Ongoing
Advanced bladder, prostate, lung, upper gastrointestinal	I.v. administration with docetaxel	I/II	Ongoing
Advanced melanoma, lung, ovarian	I.v. administration with paclitaxel and carboplatin	I/II	Ongoing
Metastatic cancers including head and neck	Local therapy in combination with radiation	II	Ongoing
Various metastatic cancers	Local therapy in combination with radiation	I	Phase Ia complete; phase Ib ongoing
Recurrent malignant glioma	Intratumoral infusion as monotherapy	I/II	Ongoing
Various metastatic cancers	I.v. administration as monotherapy	I	Complete*
Various metastatic cancers	I.v. administration as monotherapy	I	Complete**
T2 prostate cancer	Local administration	I	Complete***
Various subcutaneous tumors	Local administration	I	Complete#

*Of the 18 patients treated, 8 demonstrated stable disease, including a patient with progressive breast cancer who experienced a 28.5% shrinkage in tumor volume. **Reolysin® was well tolerated by patients, with several notable changes in stabilization of disease, as well as some minor tumor regressions in patients who had failed all previous treatments. ***Evidence of apoptotic tumor cell death in 4 of 6 patients, with no safety concerns; 1 patient experienced a PSA drop of 53% and prostate gland shrinkage of 67%. #None of 18 patients receiving Reolysin® experienced any serious adverse events related to the virus, nor were there any dose-limiting toxicities; evidence of viral activity was detected in 11 of 18 patients, with the tumour regression ranging from 32% to 100%.

phase I Systemic Administration Trial showed that Reolysin® delivered systemically was well tolerated and active in patients with colorectal, bladder, prostate, pancreatic, endometrial and non-small cell lung (NSCLC) cancers. A phase II trial to evaluate i.v. Reolysin® in patients with various sarcomas that had metastasized to the lung demonstrated that at least 1 patient of the first 38 patients treated experienced a response and stable disease for longer than 6 months (54).

During a phase I trial of i.v. Reolysin® pretreatment of patients with advanced cancers, a detailed analysis of the immune effects was conducted by collecting samples for analysis of peripheral blood lymphocytes. $CD3^+CD4^+$ cells (helper T lymphocytes) were reduced in most patients, but after reovirus therapy their numbers increased in 47.6% of patients. Most patients had high baseline $CD3^+CD8^+$ cell (cytotoxic/suppressor T lymphocyte) levels, with 33% showing incremental increases after therapy. Most patients had high numbers of circulating $CD3^-CD56^+$ cells (NK cells) before therapy and in 28.6% this increased with treatment. $CD3^+CD4^+CD25^+$ (regulatory T cells that suppress antitumor immunity) were largely unaffected by the therapy. These data confirm that even heavily pretreated patients are capable of mounting dynamic immune responses during treatment with Reolysin® (61).

Several clinical trials, which included over a hundred patients, demonstrated that intratumoral (including intracranial and intravenous) administration of Reolysin® was well tolerated (47, 54-60).

Future directions

The study of the interactions of reovirus with chemo- and/or radiotherapy will be a necessary step in its therapeutic development. In addition to the drug combination used, the timing of drug and virus aplication also appears to be important. Our unpublished results showed that reovirus applied after cyclophosphamide is much more effective than reovirus injected before cyclophosphamide in syngeneic tumor models. Additionally, it will be very interesting to ascertain whether combination therapy with multiple OVs will improve therapeutic efficiency. Since OVs act through different cellular targets, it is probable that tumors resistant to one virus may be sensitive to others. Also, antiviral immunity inhibiting one type of OV could be overcome by using another type of virus (27). The production of neutralizing antibodies could be suppressed by administration of anti-CD20 antibodies (rituximab) prior to the oncolytic virotherapy, or they may be eliminated by plasmapheresis (6). Complement could be transiently neutralized by administration of cobra venom factor or cyclophosphamide (62).

In conclusion, reovirus appears to be a safe and promising anticancer therapy in tumors with activated *RAS* and may become a part of combination therapy.

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Source

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In vitro and *in vivo* effects of reovirus on HPV16-transformed mice cells

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Oncolytic viruses are examined to serve as anticancer therapeutics. It is expected that in addition to direct oncolytic effect their action will also help eliciting a solid antitumor immunity. In presented series of experiments we have employed two HPV16-transformed mouse (strain C57/B6) cell lines, TC-1 and MK16/III/ABC (MK16), and reovirus type 3, strain Dearing (RV). Both cell lines are highly susceptible to RV and produce large amounts of infectious virus *in vitro* while normal human are not susceptible to RV. Still, some differences were encountered. TC-1 cells produced moderately lesser amounts of infectious virus, but, paradoxically, were more efficient producers of δ1 antigen of RV and as a consequence of virus infection died more rapidly than simultaneously infected MK16 cells. Minor differences between the cell lines were observed in the percentage of cells arrested in theG2/M phase of the cell cycle and in some markers of apoptosis. When inoculating high doses ($5x10^6$) of infected cells (MOI 10 PFU/cell) into syngeneic animals their oncogenic activity was strongly suppressed, nearly completely in the case of MK16 cells and somewhat less efficiently in the case of more oncogenic TC-1 cells. Immunizing experiments in which non-oncogenic doses (10^6) of RV infected TC-1 cells were tested in parallel with the same doses of irradiated cells brought surprising results. When immunized animals were challenged with TC-1 cells, the irradiated cells proved to be a much better immunogen that the infected cells. However, when challenged with MK16 cells the opposite was true. It is believed that this difference was associated with the different biological properties of the cell lines tested.

Key words: reovirus type 3, HPV16-transformed mouse cell lines, apoptosis, cell cycle, immunization/challenge experiments.

Despite the advances in clinical oncology that help to decrease patients´ mortality, cancer remains one of the main causes of death in developed countries. Current efforts to improve cancer therapy are aimed at enhancing drug efficacy while maintaining acceptable degree of toxicity. In order to succeed, innovatory therapeutic modes have been designed. One of these is represented by oncolytic viruses that infect, replicate in, and lyse tumor cells, but do not grow at all, or at a limited extent, in non-tumor cells [1–3].

Oncolytic viruses can cause the destruction of tumor cells not only by direct lysis resulting from virus replication. They may also effectively induce antitumor immunity that comprises both the antibody and T cell responses targeting tumor-associated antigens [4–6]. Schulz et al. showed that cells infected with viruses were more effective at delivering non-viral antigens for cross-priming of dendritic cells *in vivo* [7]. Furthermore, viral infection may induce cytokine production which support infiltration of tumor microenvironment by cytotoxic cells such as macrophages, neutrophils, and NK cells. Experiments with melanoma cell lines showed that reovirus infection of tumor cells induces lymphocyte expansion, IFN-**γ** production, specific anti-tumor cytotoxicity, and activates CD8+ T cells specific against the tumor [8–11].

However, immune reactions are likely to influence the efficiency of oncolytic virus therapy in various ways and, apparently, at various levels [12, 13]. For example, immunosuppresion decreased efficiency of oncolytic herpesvirus G207 in transplanted tumors [14]. On the other hand, Hirasawa et al. [15] found an increased efficiency of reoviral therapy after application of cyclosporine A or anti-CD4 and anti-CD8 antibodies in immunocompetent-mice model. However, it still remains to be determined which of the different immune mechanisms are involved in suppression of viral growth and which contribute to the establishment of anti-cancer immunity.

Reoviruses (acronym for **R**espiratory **E**nteric **O**rphan viruses) are viruses that, thanks to their natural properties, selectively

replicate in a wide spectrum of tumor cells [16]. Research into the mechanism of reovirus tumor selectivity has revealed that they replicate well in cells with activated *ras* signaling pathway, which is an attribute shared by many cancer cells [17]. *In vitro* studies, animal experiments and, subsequently, clinical studies suggested that reovirus type 3, strain Dearing /RV/, may be an efficient and safe anticancer agent [for review see 2, 3, 18].

Based on the results of experimental studies, the immunotherapy of HPV-associated tumors seems to be an effective, highly perspective therapeutic modality for treatment of these cancers and animal models remain of particular importance in this respect [19–24]. The only HPV proteins expressed in cervical carcinomas are the non-structural proteins E6 and E7. Therefore they are considered targets for immune reactions. In addition to the papillomaviral oncoproteins, other viral antigens generated by RV in infected cells might enhance anticancer immune reactions.

In the previous paper we reported on the efficacy of RV inoculation into tumors induced by HPV16 and H-ras- transformed cells [24]. The aim of this study was to compare the effects of RV on two HPV16 and H-ras-transformed cell lines and to determine whether RV potentiates the efficiency of tumor vaccines expressing HPV16 E6 and E7 in syngeneic mouse model.

Materials and methods

Cell lines. TC-1 and MK16/IIII/ABC (MK16) cells were described in previous papers [24]. In brief, MK16 cells were established in our laboratory by transformation of C57/B6 primary kidney cells by co-transfection with E6/E7 genes of HPV16 and activated H-ras oncogene [21]. They have an epithelioid morphology and downregulated MHC class I expression. However, MHC class I molecules are formed after exposure to interferon γ *in vitro* [25] and in the course of tumor growth *in vivo,* apparently due to endogenous interferon γ production [26]. These cells do not express B7.1 molecules at their surfaces but synthesize relatively large amounts of IL-1α [27]. One TID_{50} corresponds to approximately $5x10^4$ MK16 cells. Subcutaneous tumors induced by MK16 cells do metastasize to lymph nodes and lungs. TC-1 cells were derived by co-transfection of the C57/B6 mouse lung cells with E6/E7 genes of HPV16 and activated H-ras oncogene [28]. They are of fibroblastoid morphology and express MHC class I molecules at their surfaces. Furthermore, they express co-stimulatory B7.1 molecule at surfaces [29] but do not produce appreciable amounts of IL-1α [27]. One TID_{50} corresponds to approximately $5x10^3$ TC-1 cells. Subcutaneous tumors induced by these cells do not metastasize. Vero cells and normal human fibroblasts (NHF) were kindly provided by J. Cinatl Jr (J.W. Goethe University, Frankfurt/M, Germany). All cells were cultivated in IMDM medium or RPMI-1640 medium (both Sigma Aldrich Corp., St.Louis,Mo) supplemented with 10% fetal calf serum, 1% L-glutamine and antibiotics (all PAA Labs., Linz, Austria), at 37°C either in plastic culture flasks or in Petri dishes kept in humidified atmosphere with 5%CO_2.

Reovirus. Reovirus type 3, strain Dearing, was kindly provided by J. Cinatl Jr.. The virus was propagated in Vero cells. Virus stocks were kept frozen at –80˚C. Their titres were determined by a standard plaque assay using agar overlay. RV growth curves in Vero, MK16 and TC-1 cells were constructed after infecting the cultures at a MOI of 5 PFU/ cell. Samples were taken at 0 h (i.e. at the time of withdrawing the unattached virus, washing the cultures with PBS and adding media) and then at 6, 20, 26 and 48 hours post infection. After repeated freezing and thawing, the suspensions were spun down and the supernatants were titrated in Vero cells grown in 96-well plates. The final titres were determined 7 days after inoculation.

MTT test. The cytotoxicity of RV was determined using MTT test. For constructing the dose-response curves, the cultures of Vero, TC-1, MK16 and NHF cells were infected with different doses of stock RV diluted in the growth medium. Briefly, 10^4 cells in 0.1 ml of cultivation medium harvested in their exponential growth phase were seeded into a 96-well plates. Twenty-four hrs later, equal volumes of decreasing dilutions (10^{-3} to 10^{-12}) of RV were added. After 4 day incubation, the MTT solution was added. After additional 4 hr incubation, 50% N,N-dimethylformamide containing 20% SDS was added to disolve blue formazan crystals formed in functional mitochondria. The absorbance of emerged violet solution was measured at 570 nm for every single well by ELISA reader Versamax (Molecular Devices, CA, USA). The mean absorbance of wells containing only medium was subtracted from each measurement. The mean absorbance of control wells (containing cells and medium but not RV) was considered as 100% viability and the values determined for the infected cells were calculated as the proportion of this control. Each value represented the mean of 8 wells with corresponding standard deviation (SD).

DNA analysis. NHF, TC-1 and MK16 cells were incubated as described above for 24 hrs. Then RV was added at the MOI of 5 PFU/cell. Cell cycle was monitored in infected and control non-infected cultures by measuring the DNA contents at 24, 48 and 72 hrs after infection. Cells were stained by DNA Prep Reagent Kit (Beckmann Coulter, Fullerton, CA, USA) according to manufacturer´s instructions. The fluorescence intensity of 30,000 cells was measured by FACS Calibur (BD, San Jose, CA, USA) flow cytometer and list mode data were analyzed by ModFitLT software (Verity Software House, Topsham, ME, USA). The tests were repeated four times.

Apoptosis detection. For detection of apoptosis 24, 48 and 72 hrs after infection at the MOI 5 PFU /cell, we used cytometric detection of cells with subdiploid DNA content after extraction of low molecular weigh DNA [30]. Cells were harvested by trypsinization, washed and fixed in 70 % ethanol overnight at 4°C and washed again. The cell pellets were resuspended in phosphate-citrate buffer (pH 7.8) and incubated for 30 min at room temperature. After one more centrifugation, the cell pellets were resuspended in PBS and stained for DNA content by propidium iodide with RNAse (DNA Prep Stain solution from DNA Prep Reagents Kit, Beckmann Coulter). Cells were tested using FACSCalibur flow cytometer. The analysis of cellular DNA content revealed apoptotic cells as the cells represented by a „sub-G1 peak" on DNA content frequency histogram.

Detection of reovirus antigen in infected cells. We determined RV antigen producing cells by indirect immunofluorescence measured by flow cytometry after permeabilisation of the cells using Fix & Perm kit (An der Grub, Kaumberg, Austria) according to manufacturer´s protocol. As the primary antibody we used MAB994 monoclonal antibody reactive with RV type 3 σ1 hemaglutinin (Millipore, Billerica, MA, USA) and as the secondary antibody the FITC-Conjugated Goat Anti-mouse Immunoglobulin Polyclonal Antibody (BD, San Jose, CA, USA). Non-infected cells cultivated under the same conditions were used as a negative control.

Animals and oncogenicity tests. C57BL/6 female mice, 7-8 week old, were obtained from Charles River, Germany. All work with animals was done according to the Guidelines for Animal Experimentation valid in the Czech Republic. In different experiments the mice were inoculated subcutaneously (s.c.) with either $5x10^6$ RV-infected MK16 cells, or 10^5, $5x10^5$, 10^6 or $5x10^6$ of RV-infected TC-1 cells suspended in 0.2 ml PBS. In all instances the MOI was 10 PFU/cell. Groups of mice were simultaneously inoculated with non-infected cells. Animals were inspected twice a week for up to 113 days. Tumor size was expressed as the area index (AI), as described previously [24].

Cell vaccines TC-1 cells grown in culture flasks were infected at the MOI of 5 PFU/cell. After 3 hr incubation at 37°C, the unattached virus was removed by three washes with PBS. Cells were trypsinized, washed two times with PBS and 10^6 cells were administrered s.c. to mice. The second dose was administered two weeks later. In parallel, groups of mice were inoculated with the same doses of irradiated (100 Gy) TC-1 cells. Two weeks after the second dose the animals were challenged with either $5x10^5$ MK16 cells or $5x10^4$ TC-1 cells administered at a site different from the immunization sites. Thus, approximately 10 TID_{50} of the cells were used in both instances. Non-immunized mice served as controls.

Statistics Numerical data were presented as mean and SD and analysed using Student t-test**.** Tumor formation was analysed in 2x2 contingency tables by two-tailed Fisher's exact test. Analysis of tumor growth curves was performed by two-way analysis of variance. Calculations were done using GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA). A difference was considered significant if $p<0.05$.

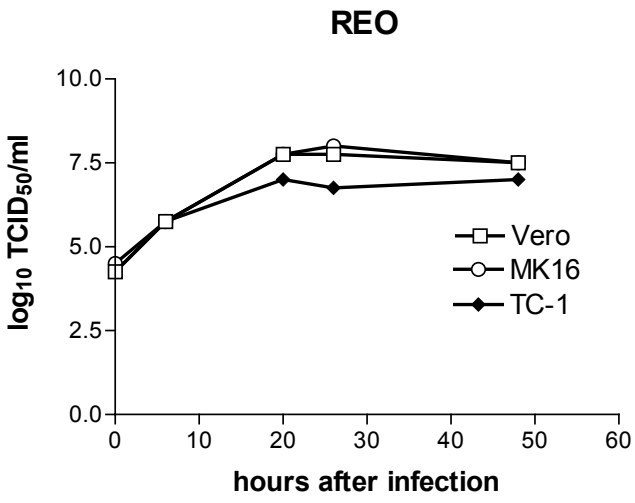


Fig.1. Growth curves of reovirus in Vero, TC-1 and MK16 cells

Results

Growth of reovirus in Vero, TC-1 and MK16 cells. Fig 1 presents the growth curves of RV in Vero, MK-16 and TC-1 cells. It can be seen that they were nearly identical in all three cell lines, but the production of infectious virus was somewhat diminished in TC-1 cells. The results of MTT are shown in Fig 2. They suggest that infected TC-1 were dying more rapidly than MK16 cells infected at the same MOI. As expected, RV did not disturbe the viability of NHF cells.

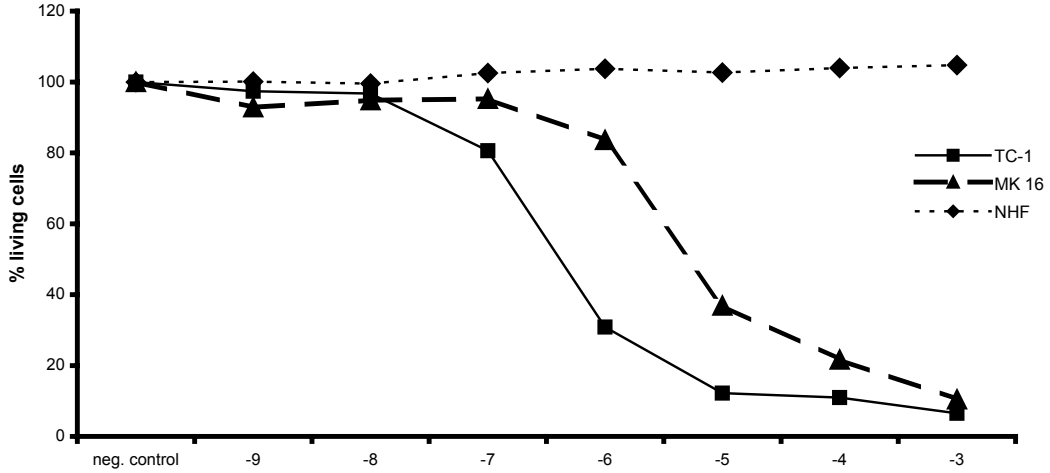


Fig.2. Results of MTT tests.

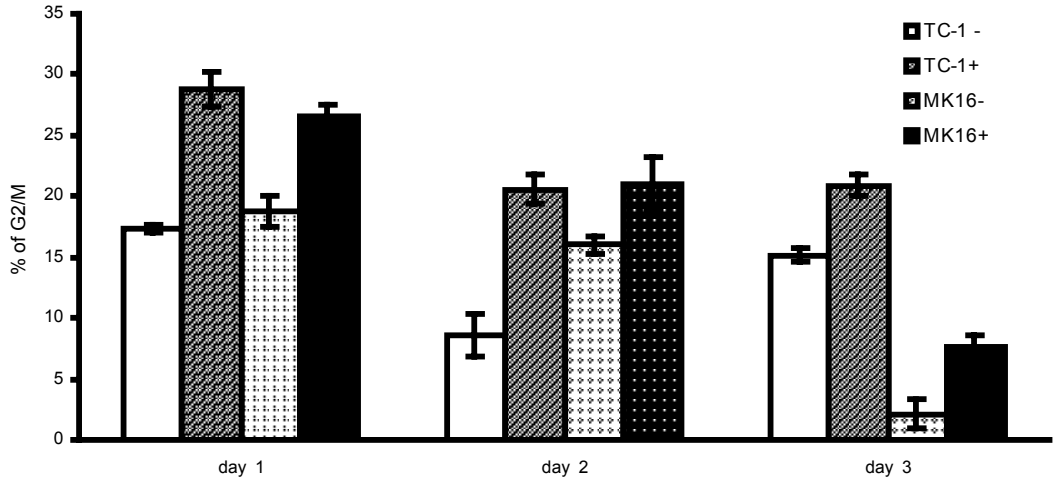


Fig.3. Percentage of cells in G2/M phase detected by flow cytometry DNA analysis: TC-1 and MK-16 cells infected (+) or not (–) with RV. (NHF not shown because percentage of G2/M phase was < 1% in all NHF samples)

Influence of reovirus on cells. As could be expected, TC-1 and MK16 cells but not NHF cells infected with RV had significantly increased proportion of cells in G2/M phase compared to noninfected cells 48 and 72 hrs ($p< 0.01$) after infection. The increase of percentage of cells in G2/M phase was higher in TC-1 cells than in MK16 at 24 as well as at 48 hrs after infection, however 72 hrs after infection, the contrary was observed (Fig 3).

As indicated in Fig. 4 we detected increased percentage of apoptotic cells 48 and 72 hrs after infection of TC-1 and 72 hrs after infection of MK16 ($p<0.01$). Percentage of apoptotic NHF in infected culture was only slightly increased.

Interesting results were obtained when the production of RV antigens in the infected cells was followed. Results expressed as means of three experiments shows Fig. 5. There were marked differences between TC-1 and MK16 infected cells. On the first day more than 75% of TC-1 cells and on the second and the third day more than 90 % cells produced detectable amounts of RV antigen. On the other hand, only 15 %, 26.6 % and 44.5 % of MK16 cells were positive on the first, second and third day, respectively. As expected, there was no marked increase of RV positive cells with time in the cultures of nonpermissive NHF cells (16.5% on the first; 11.5% on the second and 20.2% on the third day, respectively). This

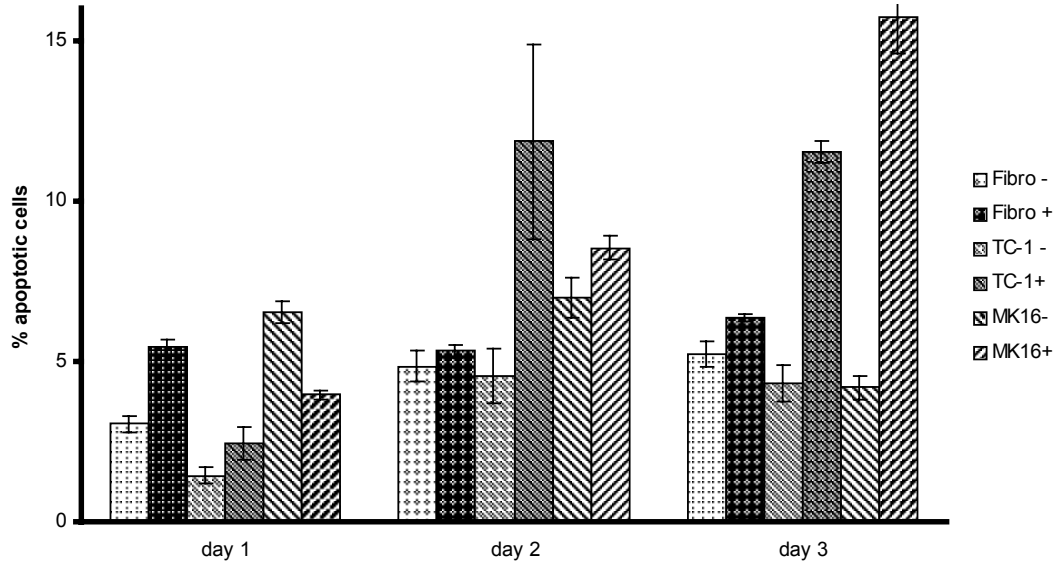


Fig.4. Percentage of apoptotic cells detected by flow cytometry as subdiploid peak: NHF, TC-1, MK16 without and with RV (– and + respectively) .

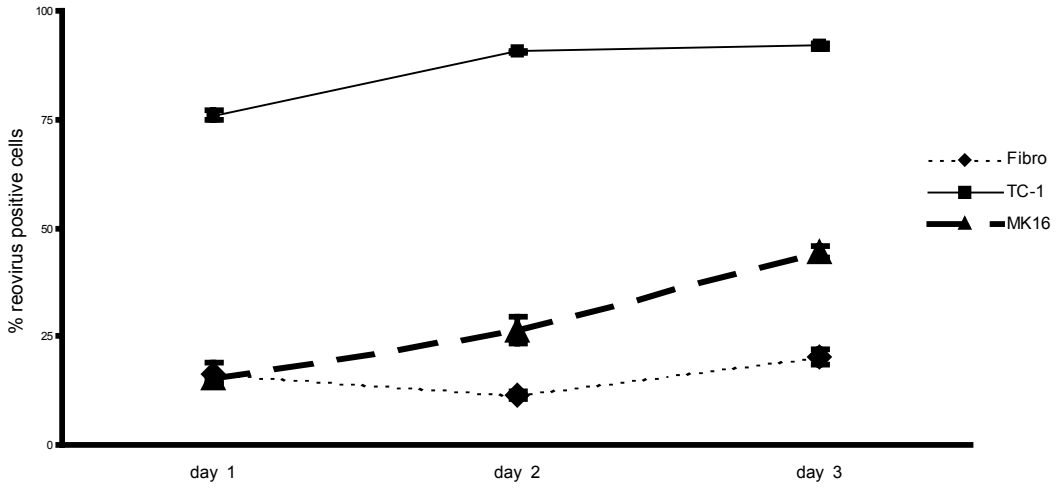


Fig.5. The production of reovirus antigens in the infected cells. Indirect immunofluorescence measured by flow cytometry - primary antibody MAB994 monoclonal antibody anti reovirus type 3 σ1 hemaglutinin.

implies that an abortive virus infection occurred in a fraction of the NHF cell population

. *Pathogenicity of reovirus-infected cells.* The oncogenicity of $5x10^6$ TC-1 and MK16 cells which were either infected with RV (at the input MOI of 5 PFU/cell) or remained non-infected is shown in Fig 6. It can be seen that all animals inoculated with non-infected cells developed tumors. In agreement with previous experiments, in these mice the tumors were detected earlier in the TC-1-inoculated mice than in those inoculated with MK16 cells. Five of 6 animals to which RV-infected TC-1 cells had been administered developed tumors. However, these tumors appeared significantly later than in mice inoculated with non-infected cells, and one of these regressed. The other tumors grew progressively. In the case of MK16 cells the tumor suppressive effect of infection with RV was nearly complete. Although tumors developed in four animals around day 50 after inoculation, they were of small size (1 - 2 mm^3) and regressed completely in the course of subsequent weeks and all animals remained tumor-free until the end of the observation period. In the subsequent experiment lower doses, 10^6, $5x\ 10^5$ and 10^5 of RV-infected TC-1 cells were tested. In this experiment all animals remained tumor-free till the end of the observation period (results not shown).

Immunogenicity of reovirus-infected cells. To find out whether the RV-infected HPV16-tranformed cells were capable of inducing immunity in the inoculated animals, mice were immunized with either RV-infected or irradiated TC-1 cells and challenged with 10 TID_{50} of either TC-1 or MK16 cells. The results of the challenge experiment shows Fig 7. It can be seen that both the infected and irradiated cells induced significant protection against TC-1 cells. However, this was much more pronounced when irradiated cells were used. Four out of six animals immunized with irradiated cells remained tumor-free. On the other hand, all animals immunized with the RV-infected cells developed tumors, though at a slower rate. The difference between these two groups was highly significant ($p<0.001$) (see Fig 7A).

The results of the challenge with MK-16 cells were different (see Fig 7B). In this case half of the mice immunized with the infected cells developed tumors while nearly all the animals immunized with the irradiated cells did so, although

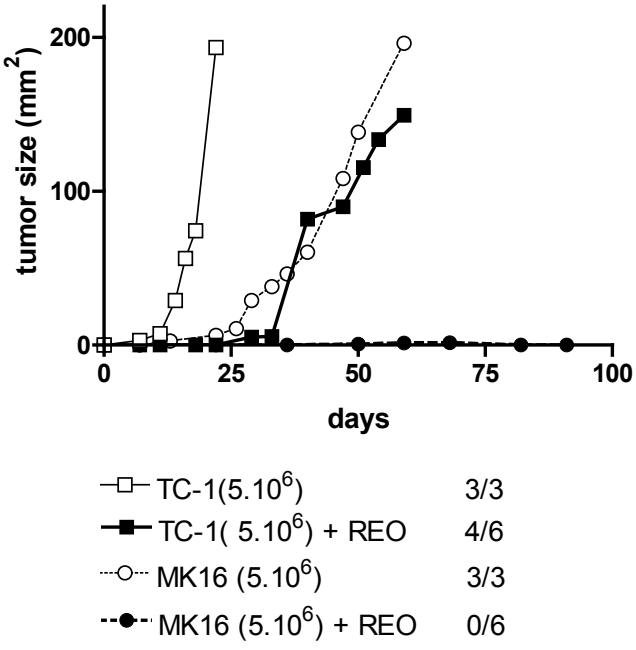
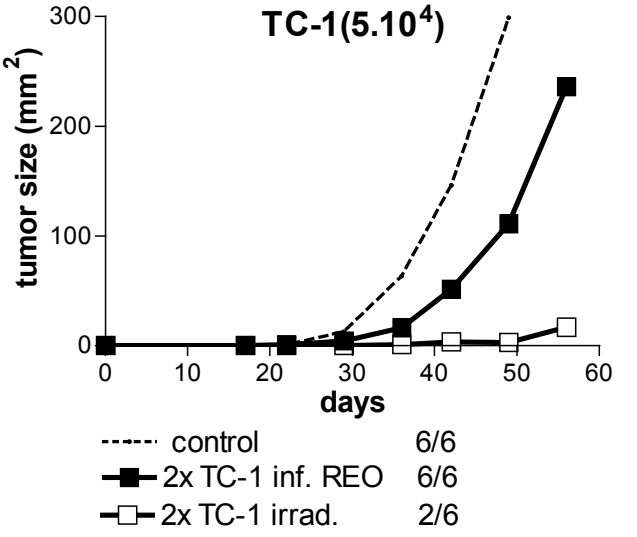


Fig.6. Tumor growth of reovirus infected and non-infected TC-1 and MK16 cells.
TC-1 + REO *vs.* TC-1 ($p<0.0001$)
MK16 + REO vs. MK16 ($p<0.0001$)



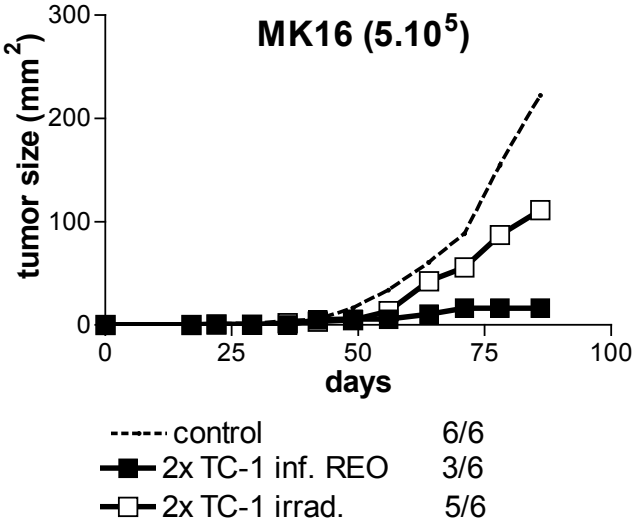


Fig.7A. Tumor growth in immunized and nonimmunized mice after challenge with TC-1 cells.
2x TC-1 inf. REO *vs.* nonimmunized (TC-1) p<0.02
2x TC-1 irrad. *vs.* nonimmunized (TC-1) p<0.001
2x TC-1 inf. REO *vs.* 2x TC-1 irrad. p<0.001

Fig.7B. Tumor growth in immunized and nonimmunized mice after challenge with MK16 cells.
2x TC-1 inf. REO *vs.* nonimmunized p<0.0001
2x TC-1 irrad. *vs.* nonimmunized p<0.02
2xTC-1+ REO *vs* 2x TC-1 irrad. p<0.01

the tumors in these animals grew more slowly than in the non-immunized controls. The difference between the controls and immunized animals was more significant with the virus infected than with the irradiated cells (p<0.0001 vs. p<0.02) .The difference between animals immunized with the infected cells and those immunized with irradiated cells was significant (p<0.01).

Discussion

In the present study we demonstrated that RV grew well in both HP16- and H-ras- transformed mouse cell lines (TC-1 and MK16 cells), but not in the control NHF cells. However, some differences between the two HPV16-transformed cell lines were apparent. Although TC-1 cells appeared to be somewhat less efficient producers of infectious RV, they were more susceptible to the killing effects of the virus and produced RVσ1 antigen in a higher percentage of cells than the MK16 cells . In accordance with the observations made in mouse L929 cells and other cell lines susceptible to RV [31, 32], the virus induced apoptosis and cell cycle arrest in G2/M phase in both TC-1 cells and MK16 cells. Although some differences between the two cell lines were encountered in repeated tests, they were not very marked. Still, they indicated that the onset of G2/M arrest and apoptosis was fairly quicker in TC-1 cells. This seems to be in consent with the findings mentioned above. Such difference was observed in all repeated tests.

The influence of RV on the viability of both the MK16 and TC-1 cells was manifested by a decreased oncogenic ability in syngeneic mice. When using high dose ($5x10^6$) of cells infected at the MOI 10 PFU/cell, 4 out of 6 mice inoculated with the infected TC-1 cells developed progressively growing tumors while all animals inoculated with similarly infected MK16 cells were tumor-free still at the end of the observation period. This definitely does not mean that TC-1 cells were less susceptible to the killing effects of RV than the MK16 cells. Such a conclusion would be in disagreement with the results of the *in vitro* tests. Two other factors might be involved. First, TC-1 cells are more oncogenic than the MK16 cells. Thus, the dose inoculated corresponded to approximately $5x10^3$ TID_{50} in the case of TC-1 cells but only to $5x10^2$ TID_{50} in the case of MK16 cells. Should the same fraction of the cells survive in both the TC-1 and MK16 cells, one would expect a residual oncogenic activity in the former rather than in the latter cells. This is what actually happened. Second, the incubation period between cell inoculation and tumor development is longer in the latter than in the former cells. Thus, in the case of MK16 cells there was more time for establishing immunity elicited by the infected cells present in the inoculum.

The most important aim of our study was to find out whether the presently used tumor cells when infected with RV would represent a more potent immunogen than the non-infected irradiated cells. In the immunization experiment TC-1 cells were used and both the TC-1 cells and MK16 cells were used for the challenge. The results were rather surprising. When the homologous TC-1 cells were used, then the irradiated cells were clearly a better immunogen than the virus-infected cells. However, the situation was opposite, when the immunized animals were challenged with MK16 cells: the immunity induced by the infected TC-1 cells appeared to be much more solid than that

induced by the irradiated cells. The reasons for this difference are not clear at this moment. One can only speculate that it reflects the differences in the biological properties of the two cell lines as described above and, possibly, some other not yet known. Although both cells share HPV16 E6 and E7 antigen, it is likely that the two cell lines differ in their antigenic make-up [33] which may, in addition, be influenced by the virus infection. Experiments are under way to clarify the mechanisms involved. Without respect to their nature, it seems clear from the present data that the assumed potentiation of the immunogenicity of the tumor virus-infected cells may indeed come into force in some (MK16) but not in the other systems (TC-1).

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Anticancer efficiency of Reovirus in normoxia and hypoxia.

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Summary

Oncolytic viruses infect, replicate in, and lyse tumor cells but spare normal ones. One type of oncolytic virus is a naturally occurring replication competent Reovirus /RV/, which preferentially kills tumor cells with activated *Ras* signaling pathways. The aim of this study was to survey effects of RV on brain tumor derived cells *in vitro* under hypoxic conditions since hypoxia causes resistance to radio- and chemotherapy.

This study demonstrates that RV replicates preferentially in tumor cells and that the virus is able to overcome cellular adaptation to hypoxia and infect and kill hypoxic tumor cells. RV can both replicate in a hypoxic tumor microenvironment and can cause cytophatic effect and subsequently induce cell death. We found that a large proportion of cells are in hypoxia (1% O_2) killed by caspase independent mechanisms. Furthermore, we learned that cell death induced by RV in hypoxic conditions is not caused by autophagy.

Key words: reovirus, hypoxia, apoptosis, autophagy, glioblastoma, medulloblastoma

Introduction

Despite advances in modern medicine, cancer remains one of the main causes of death in all developed countries. Current efforts to improve cancer therapy are attempting to enhance drug efficacy, while maintaining acceptable toxicity levels. In order to succeed in reducing the number of cancer related deaths, novel therapeutics have been designed to target tumor- specific attributes in order to permit higher doses with fewer side effects. One such example is oncolytic viruses.

Oncolytic viruses /OV/ infects, replicate in, and lyse tumor cells but spare normal ones. Most OV are prepared by genetic engineering for tumor selectivity, though there are a few naturally occurring ones *e.g.* reovirus. Replication of OV in tumor cells can increase their effect. However, physical barriers such as necrotic areas, stromal cells, extracellular matrix or basal membrane, may limit the spreading of the virus. OV can mediate the destruction of tumor cells using more mechanisms than by merely direct lysis caused by viral replication; they may induce antitumor immunity and some viruses express cytotoxic proteins (Ring 2002). On the other hand, OV induces an anticancer immune response that limits their effect (Smith and Chiocca 2000). Clinical studies were performed with OV, including reovirus (Reolysin) (Eckschlager and Figova 2008)

Reovirus /RV/ (Respiratory Enteric Orphan virus) is a replication-competent, naturally occurring virus that preferentially kills tumor cells (Coffey et al. 1998). RV replicates in the presence of an active *Ras* signaling pathway, which is common in cancer cells (Strong et al. 1998a). In humans, RV is isolated from the respiratory and gastrointestinal tract but they are not associated with any disease - orphan viruses, a type of virus that is not associated with disease. (Tyler et al. 2001).

The RV lytic cycle consists of several steps. It begins with the attachment of virion to the receptor of the host cell, followed thereafter by receptor-mediated endocytosis. Within the endosome, proteolysis of viral outer capside proteins gives rise to an intermediate subviral particle (ISVP). Receptor binding and disassembly must occur within the same cellular compartment to elicit an apoptotic response. A critical component of the signaling cascade that leads to apoptosis of RV-infected cells is the transcription factor NF-κB (O'Donnell et al. 2006). RV also activates c-Jun N-terminal kinase and extracellular signal-related kinase (Clarke et al. 2001), but their involvement in apoptosis induction is not understood. Triggered ISVPs then penetrate through endosomal membrane. Afterwards, transcription of 10 RNA

segments mediated by viral ds RNA-dependent RNA polymerase proceeds. Later, synthesis of minus RNA strands occurs and secondary transcription of late viral mRNAs begins. Final composition of the outer capsid yields virus particles (Norman K.L. and Lee P.W.K. 2001) However, viral transcription is not indispensable, as inhibitors of viral RNA synthesis do not diminish the capacity of RV to induce apoptosis (Connolly and Dermody 2002).

The mechanism of RV tropism in transformed cells is a defective cellular anti-viral response in *Ras*-pathway transformed cells. In normal cells, present reoviral dsRNAs activate PKR, which in turn phosphorylates alpha subunit of initation factor 2, eIF-2α. The phosphorylation shuts off any further protein translation and thus inhibits the initiation of translation of viral transcripts. In cells with an activated *Ras* pathway, the phosphorylation of eIF-2α is inhibited, resulting in viral translation and subsequent entrance into the viral lytic cycle (Strong et al. 1998b). RV compels cell cycle arrest at G1 and G2/M, induces apoptosis and activates MAPK cascades. RV-induced apoptosis involves members of TRAIL and is associated with the activation of both death receptor and mitochondrial-associated caspases (Smakman et al. 2005).

Hypoxic areas are frequent in solid tumors, as a consequence of pathological microcirculation within the tumor and by the poor quality of the tumor vessels (Vaupel et al. 1989). Notably, hypoxia-induced resistance is not limited only to conventional chemotherapy but it can also decrease the efficiency of targeted therapy, as documented with imatinib in cases of chronic myeloid leukemia (Giuntoli et al. 2006). Additionally, hypoxia induces genomic instability that leads to progressive transformation of cancer cells into more malignant phenotypes (Huang et al. 2007). The presence of hypoxic regions correlates with more aggressive phenotypes, lower response rates and a decline in overall disease survival (Hockel et al. 1999).

The aim of this study was to survey effects of RV on brain tumor derived cells *in vitro* in hypoxic conditions.

Materials and Methods

Cell lines and virus

Human glioblastoma cell line U373, medulloblastoma cell line Daoy as well as non-transformed human fibroblasts. Mouse L929 cell line was used for virus cultivation and titration. Cells were cultivated in IMDM supplemented with 10% foetal bovine serum, 1% glutamine, streptomycin and penicillin (PAA Laboratories, Pasching, Austria). The cells were cultured at 37˚C in 5% CO_2 under normoxia (21% O_2). Hypoxia (1% O_2) was generated in a closed system - hypoxic chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA) with a defined gas mixture containing 1% O_2, 5% CO_2 and 94% N_2.

RV serotype 3 Dearing was purchased from ATCC (LGC Standards Sp. z.o.o., Lomianki, Poland) Reovirus serotype 3 was purified according to the protocol of Smith et al. (Smith et al. 1969)with the exception that 2-mercaptoethanol was ommited from the extraction buffer.

L929 cells grown in 6-well plates to 80% confluency were infected with RV at an MOI of 20. After 72 hours of incubation, the cells and supernatants were subjected to three freeze-thaw cycles and centrifuged to omit cell debris. Viral titre was determined by plaque assays using the L929 cells. The homogenous virus was aliquoted and stored at –80 ˚C. A new aliquote was always used for each experiment.

Immunoblotting

Cells were washed with PBS, trypsinised with 0.05% trypsin and counted. 2,5 million cells containig pellets, washed two times with PBS, were stored at –80 ˚C. Thawn pellets were used for preparation of whole cell extracts. Protein levels in cell lysates incubated on ice were measured using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with serum albumin as a standard. Equal amounts of protein were electrophoresed on a 11%, 16% (for LC3 protein) and 6% (for HIF-1α protein) polyacrylamide gel. Proteins were transferred by wet electroblotting to a nitrocellulose membrane (Bio-Rad Laboratories). Non-specific binding was blocked in 5% non-fat milk in PBS with 0.1% Tween-20 or TBS with 0,1% Tween-20 and 1% BSA for LC3-II. The membranes were then exposed to rabbit polyclonal antibody to HIF-1α (Upstate Biotechnology, Lake Placid, NY) dilution of 1:3000, the mouse monoclonal antibody to β-actin (Abcam, Cambridge, MA) at a dilution of 1:3000 or the polyclonal anti-reovirus antibody that was prepared for us by immunization of rabbits (Seva-Imuno Prague, Czech Republic) was used at a dilution of 1:200 000. The appropriate HRP-

conjugated secondary anti-mouse or anti-rabbit antibody (Bio-Rad) was used at a dilution of 1:2000. Antibody binding was visualised by enhanced chemiluminescence following manufacturer´s instructions.

Flow cytometry analysis

Cell cycle analysis. Cell cycle distribution of cells exposed to ambient or hypoxic conditions was evaluated. U373 and Daoy cells were either infected with RV at an MOI of 10 or left uninfected and exposed to either ambient oxygen concentration or 1% oxygen for 12, 24, 48, 72 and 96 hours. Cells were washed with PBS, trypsinized and the washed cell pellet was resuspended in PBS containing 10^6 cells/ml. Cells were stained by propidium iodide using the DNA Prep Reagent kit (Coulter Immunology, Hialeah, FL) according to the manufacturer's protocol; the samples were measured with FACSCalibur cytometer (Beckton Dickinson, San Jose, CA), and the data were analyzed by software ModFit LT (Verity Software House Inc., Topsham, ME).

Apoptosis quantification. At various time points, cells cultivated in the presence or absence of RV in ambient or hypoxic conditions were harvested. Apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit according to manufacturer instructions (Biovision, Mountain View, CA, USA). Cells were washed in PBS and resuspended in a binding buffer, incubated with Annexin V and propidium iodide for 10 min at room temperature and then analyzed using flow cytometry (FACSC alibur, BD, San Jose, CA, USA). Data obtained from flow cytometry were evaluated using the technique described by Bossy-Wetzel ((Bossy-Wetzel and Green 2000)).

RV antigen producing cells. These cells were detected by indirect immunofluorescence measured by flow cytometry after permeabilisation of the cells using Fix & Perm kit (An der Grub, Kaumberg, Austria) according to the manufacturer´s protocol. As the primary antibody, we used MAB994 monoclonal antibody that reacts with RV type 3 σ1 hemaglutinin (Millipore, Billerica, MA, USA); as the secondary antibody we used the FITC-Conjugated Goat Anti-mouse Immunoglobulin Polyclonal Antibody (BD, San Jose, CA, USA). As a negative control we used non-infected cells cultivated under the same conditions.

Cell viability MTT test

After the desired period of time to permit the cells to grow in different concentrations of RV in microtiter plate, the MTT solution (Sigma-Aldrich, Prague, CZE) (2 mg/ml PBS) was added, the plates were incubated for 4 hours and cells lysed in 50% N,N-dimethylformamide (Sigma-Aldrich) containing 20% of SDS (LifeTechnologies, Prague,

CZE) pH 4.5. The absorbance at 570 nm was measured for each well by the multiwell ELISA reader Versamax (Molecular Devices, CA, USA). The mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of the control cells. Each value is the mean of 8 wells with a standard deviation. The IC50 values were calculated from the linear regression of the dose-log response curves by SOFTmaxPro.

Caspase-3 assay

Using Biovision Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Milpitas, CA, USA) level of Caspase-3 was detected according to manufacturer's instructions. Cells were lysed in a cell lysis buffer and placed in microplate together with caspase-3 substrate. After incubation for 2 h at 37^{o}C, the absorbance was measured, the background reading from cell lysates and buffers was substracted, and fold-increase in Caspase-3 activity was determined by comparing these results with the level of the uninduced control.

Statistics

All experiments were performed at least in triplicates. All numerical data were presented as mean ± standard deviation and were analyzed statistically using Student's t-test**.** P values of less than 0.05 were considered significant. Software SPSS version 10.1 was used for statistical calculations.

Results

***In Vitro* Reovirus Infection.**

To determine the susceptibility of human glioblastoma and medulloblastoma cell lines to RV compared to its effect on healthy human fibroblasts, we infected these cells with RV at an MOI of 10. As shown in **Fig. 1C** no morphological changes were detected in the normal human fibroblasts cell line, even at 96 h after infection. In contrast, all of the tumor cell lines infected with RV exhibited meaningful cytopathic effects *i.e.*,rounding and clumping of cells. **(Fig**. **1 A, B)** At 96 h postinfection, nearly 85% of all cancer cell lines examined were destroyed, as examined by optical microscopy.

Hypoxia induces expression of HIF-1α

The HIF-1α expression was evaluated to verify whether there was hypoxic phenotype established in cell lines subjected to hypoxia. HIF-1α is a transcription factor that mediates adaptive responses to changes in tissue oxygenation that is degraded in the presence of oxygen while in hypoxic conditions is stabilised. (Metzen and Ratcliffe 2004, Semenza 2010). In all cancer cell lines used in this study, HIF-1α protein expression was increased in hypoxic

condition (1% O_2) compared to ambient conditions (21% O_2)**.** The results indicate that hypoxic conditions used in all experiments incited physiological response and did not reduce cell viability. It also suggests that in cell lines used in this experiment RV decreases the degradation of HIF-1α and the effect of RV and of hypoxia is cumulative see **Fig. 2**

Hypoxia does not reduce reoviral oncolysis in hypoxic cells

Since RV naturally colonizes gastrointestinal and respiratory tracts, it is exposed to certain level of oxygen pressure. However, the conditions within a tumor mass differ with oxygen concentrations approximating 10 mm Hg or lower. (Hockel and Vaupel 2001). It has been stated that this decline in oxygen concentration negatively affects adenoviral (Pipiya et al. 2005) and VSV replication (Hwang et al. 2006). The aim of those experiments was to determine whether hypoxia does not affect the replication of wild type RV and thus reduces its oncolysis.

We evaluated the progression of RV infection using immunofluorescence microscopy and flow cytometry to compare the numbers of efficiently infected (producing RV proteins) cancer cells under ambient and hypoxic (1% O_2) conditions. There was no significant difference between hypoxic and ambient samples infected with RV during the entire experiment, and the number of positively stained cells augmented with time (data not shown). We then determined by immunoblotting whether there was a difference in viral protein accumulation in normoxic and hypoxic cells. Levels of u-viral protein appeared to be similar in normoxic and hypoxic infected cells **Fig. 3 A**

The cytopathic effects of RV under ambient or hypoxic conditions were compared, also using MTT test. The percentage of surviving cells at various time points (12, 24, 48, 72, 96hours) after RV infection did not differ significantly between hypoxic and ambient samples. **Fig. 3 B, C.**

Mode of tumor cell death following RV infection differs in hypoxic and normoxic conditions

After we learned that RV is capable of killing cells in hypoxic as well as in normoxic conditions, we measured the proportion of apoptotic cell death in these samples. Using cytometric detection of AnexinV and propidium iodide binding we detected that significantly more infected cells died of apoptosis in normoxia than in hypoxia.

To test our findings, prior to RV infection we treated cells with pan-caspase inhibitor ZVAD-fmk to block apoptosis. ZVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-

fluoromethylketone) is a pan caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases and inhibits induction of apoptosis (Slee et al. 1996) We proved that the concentrations of ZVAD-fmk we used were harmless for the cells in previous experiments (data not shown). At various time points post RV infection, we measured cell viability using flow cytometry. As a positive control for apoptosis, we used cells incubated with staurosporine, a non-selective protein kinase inhibitor that blocks many kinases through the prevention of ATP binding to them and thus initiate apoptosis (Chae et al. 2000) We found that although the viability is the same in hypoxia and normoxia during the whole experiment, the levels of apoptosis significantly differ. **Fig 4 E, F.** There was a higher rate of early apoptosis (AnexinV+ propidium iodide- cells) after RV infection in normoxia compared to corresponding hypoxic samples. However, after adding ZVAD-fmk to samples prior to RV, the level of apoptosis changed dramatically. ZVAD-fmk blocked apoptosis in normoxic samples completely while in hypoxic samples it did not. There still remained some level of apoptosis in hypoxia which seemed to be caspases independent.

Caspase-3 assay

To confirm our findings about activated caspase cascade, we performed a caspase-3 assay. As a positive control we used cells incubated with CDDP (10uM), which is a well-known inducer of apoptosis via sequential activation of caspases. We found out that the levels of induced caspase-3 did not differ significantly in hypoxia and normoxia. After adding RV to the cells, there was an increased activation of caspase-3 in normoxic but not in hypoxic conditions see **Fig. 5**

Is autophagy the type of death reovirus induces in hypoxia?

To find out the different mode of RV induced cell death, we measured the levels of LC 3-II in the samples to detect autophagy in our experimental systems. During autophagosome formation, LC3-I covalently links to phosphatidylethanolamine and is incorporated into autophagosome membranes where it recruits cargo. This lipidation process converts cytosolic LC3-I into the active, autophagosome membrane-bound form, LC3-II. Therefore, detection of conversion of soluble LC3-I to lipid bound LC3-II depicts autophagy. As a positive control, we used the sample where cells were incubated with chloroquine that arrests autophagy and induces accumulation of autophagic vacuoles (Geng et al. 2010)

We found no significant difference in normoxic and hypoxic samples nor in the samples infected with RV compared to non-infected ones see **Fig 6**; similar results were in U373 cells. This means that the low level of autophagy induced by RV after 48 hours in hypoxia is similar to that in the normoxic non-infected sample, while cells incubated with

chloroquine showed massively increased level of converted LC 3-II. Autophagy is, therefore, not the type of death that RV induces in hypoxia.

Discussion

We have shown that RV replicates preferentially in tumor cells. This is due to fact that many cancer cells have a defect in the Ras transcriptional pathway, which makes them susceptible to RV (Marcato et al. 2007; Smakman et al., 2005). Normal cells, however, by not giving a virus chance to replicate effectively, are able to suppress viral infection even after RV has entered the cell. The differences between cancer and normal cells create a wide potential of therapeutic uses of RV as anticancer agent.

We have shown that RV is able to overcome cellular adaptation to hypoxia and infect and kill tumor cells. It can not only replicate in a hypoxic tumor microenvironment but it can also cause cytophatic effect and subsequently induce cell death. A large proportion of cells in hypoxia are killed by caspase independent mechanisms. Furthermore, we found out that cell death induced by RV in hypoxic conditions is not caused by autophagy.

Hypoxia is regarded as a negative prognostic factor for malignant tumors because it causes resistance to radio- and chemotherapy (Shannon et al. 2003, Um et al. 2004). Therefore are intensively searched agents that are efficient both in normoxia and hypoxia. Hypoxia induces resistance by both HIF-1-dependent and -independent mechanisms (Rohwer and Cramer 2011). The actual contribution of different transcription factors to hypoxia-induced apoptosis resistance depends on several factors (e.g. cell type, severity and length of hypoxia, type of proapoptotic stimuli). We have shown important role of HIF-1α in NBL cell lines in experiments with HIF-1α inhibition (H.Marikova: Significance of HIF-1α expression in neuroblastoma cell lines. Diploma thesis. Institute of chemical technology, Prague, 2011) . The role of HIF-1α as an anti- or pro-apoptotic transcription factor is still discussed (Piret et al. 2002) and constitutive expression of HIF-1 α has been shown to restrict RV replication (Cho et al. 2010). We did not detect a decrease of HIF-1α caused by RV in brain tumors derived cell lines as it was described in different carcinoma cells (Cho et al. 2010). A possible explanation of this disparity may be explained by the different biology of tested cells or use of concentration of oxygen- Cho et al. used 2% of O_2 whereas we used 1%.

Preclinical studies have shown that RV proliferates only in tumor cells with activated gene of *Ras* family or its pathway that could be found in 60–80% of human malignancies. Oncolytics Biotech Inc. is currently guiding clinical studies with RV- Reolysin®. Completed

studies, which included more than a hundred patients, demonstrated that intratumoral, including intracranial and intravenous application of Reolysin®, is being well tolerated by patients. Also tested was a combination of Reolysin® with chemotherapy and/or radiotherapy (Figova et al. 2006) All of these studies have demonstrated the potential value of RV as an anticancer agent. We have shown an additional advantage of the oncolytic RV, namely its ability to replicate in an hypoxic tumor microenvironment. Its ability to replicate and cause cytopathic effect in hypoxia not diminished at all; on the contrary, even after pan-caspase block it is still able to cause apoptosis. Thus, RV is able to induce cell death independently of caspases. We have shown that it is not autophagy that RV evolves in hypoxia. Though we still do not know which type of cell death it is, this information is, however, a useful tool in work with RV in treating hypoxic neoplasms. If we are able to support its ability to induce cell death in hypoxic conditions in a special manner, we have a new, powerful tool to defeat cancer.

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Figure legends

Figure 1. *In Vitro* RV Infection. Human fibroblasts, Daoy and U373 cells were treated with reovirus (MOI = 10) and after 96 hours observed for cell viability and cytopathic effect under light microscopy.

Figure 2. Hypoxia induces expression of HIF-1α. The cells were infected with reovirus and placed in hypoxic chamber or incubated in normoxia. The control samples were left uninfected in normoxia. After different times post infection the cells were harvested and the expression of HIF-1α was examined by immunoblotting using anti-HIF-1α antibody.

Figure 3. Hypoxia does not reduce reoviral oncolysis in hypoxic cells. A Daoy cells were infected with reovirus (MOI = 10) for 96 hours and harvested for immunoblotting to detect reovirus replication using rabbit polyclonal anti-reovirus antibodies which recognizes reovirus proteins λ, μ and δ. **B** Daoy and **C** U373 cells were incubated with different concentrations of reovirus for 96 hours and percentage of surviving cells was detected using MTT test.

Figure 4. **Mode of tumor cell death following RV infection in hypoxic and normoxic conditions. A, B** Graph of Daoy and U373 cells viability (percentage of AnexinV and propidium iodide negative cells) 48 hours post infection**. C, D** Early apoptosis (percentage of AnexinV positive and propidium iodide negative cells) 48 hours post infection**. E, F** Prior to reovirus infection cells were treated with ZVAD-fmk inhibitor and staurosporine treated cells were used as positive control. Percentage of surviving and early apoptotic cells.

Figure 5. Caspase-3 assay. Cells were treated with RV (MOI = 10) and placed in hypoxia and normoxia. After 48 hours post infection cells were harvested to perform caspase-3 assay. Cells trated with CDDP (10uM) were used as positive control.

Figure 6. Is autophagy the type of RV induced death in hypoxia? Cells were treated with reovirus (MOI=10) and placed in normoxia or hypoxia. After 48 hours cells were harvested and the expression of LC 3-II as an indicator of autophagy was examined by immunoblotting using anti-LC 3-II antibody.

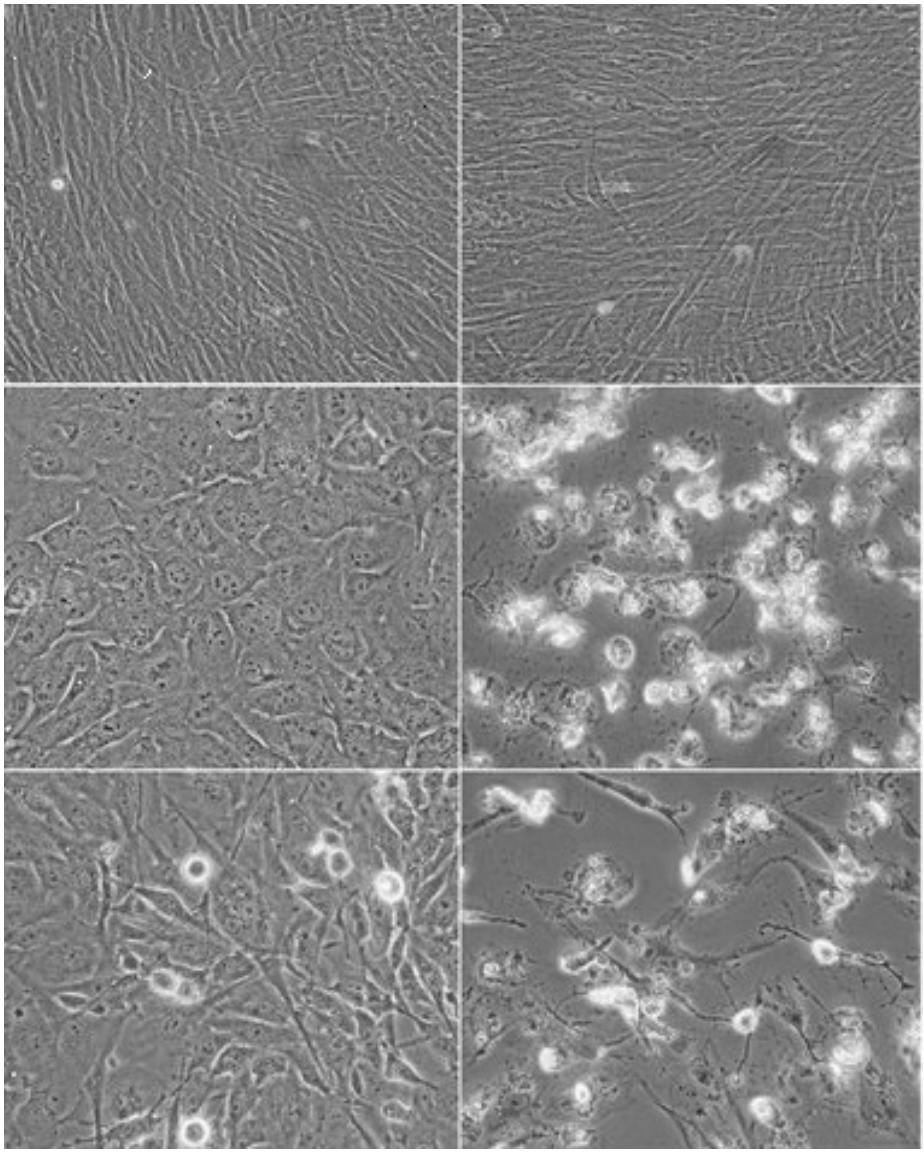

Figure 1. *In Vitro* RV Infection. Human fibroblasts (at the top), Daoy (in the middle) and U373 (at the bottom) cells were treated with reovirus (MOI = 10) and after 96 hours observed for cell viability and cytopathic effect under light microscopy.

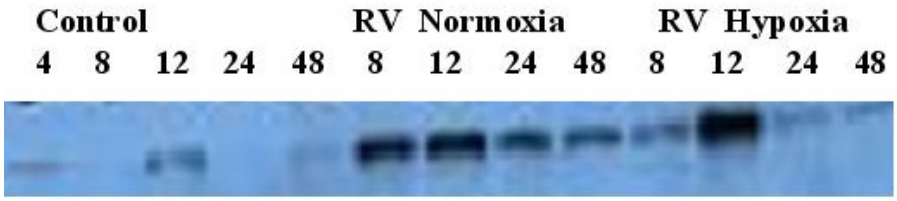

Figure 2. Hypoxia induces expression of HIF-1α.

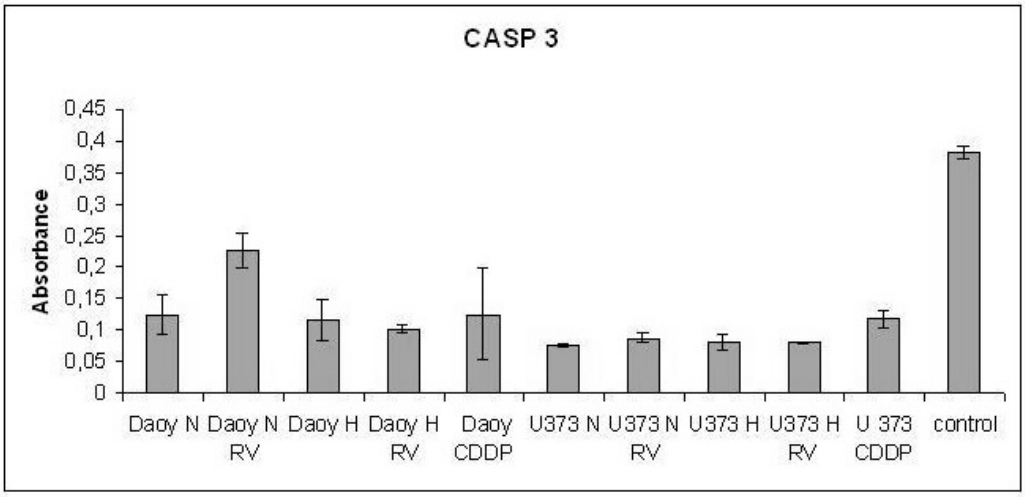

Figure 5. Caspase-3 assay.

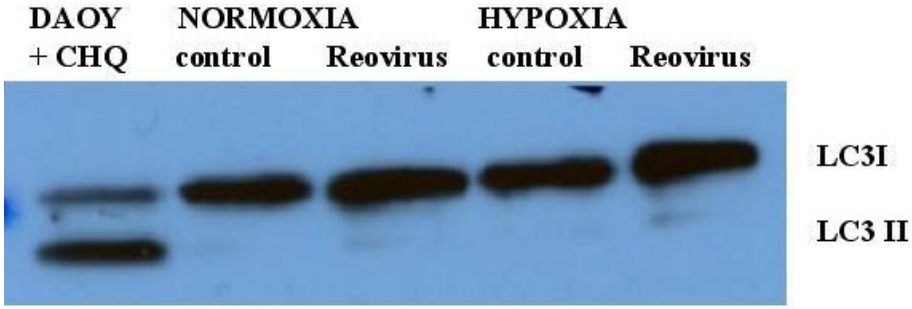

Figure 6. Is autophagy the type of RV induced death in hypoxia?

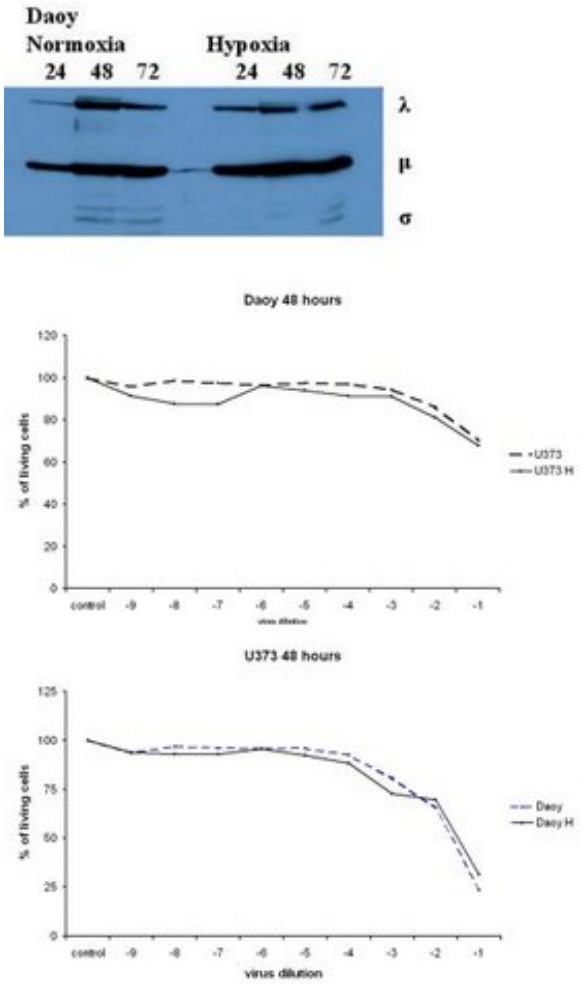

Figure 3. Hypoxia does not reduce reoviral oncolysis in hypoxic cells.

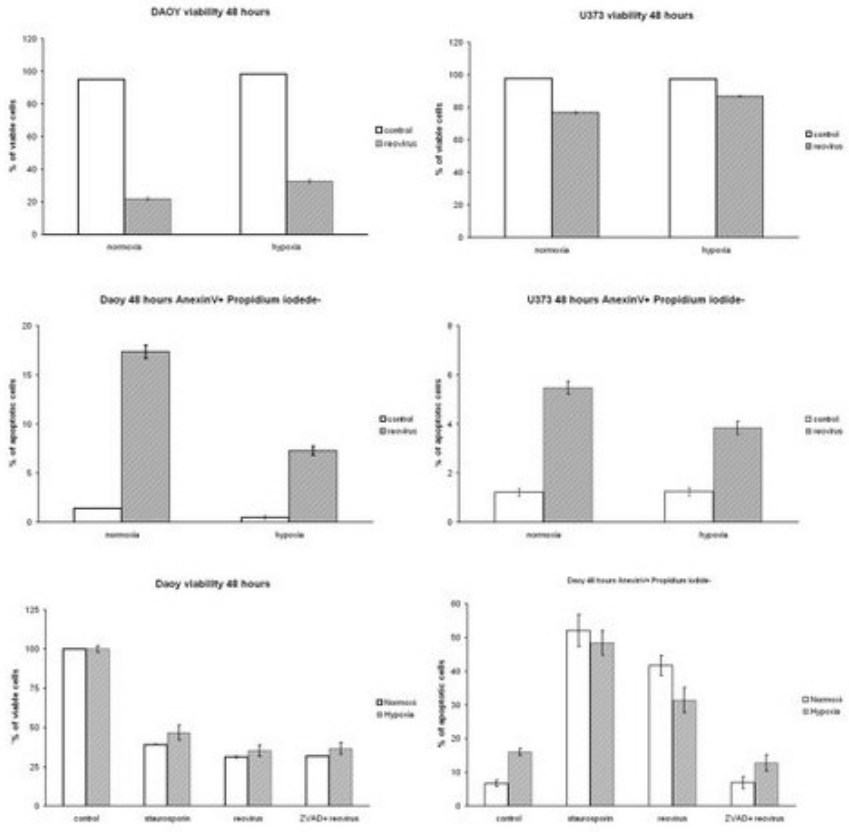

Figure 4. **Mode of tumor cell death following RV infection in hypoxic and normoxic conditions.**

6. Záver a diskusia

Intenzívny výskum v priebehu uplynulých 30 rokov od objavenia onkolytického účinku reovírusu a jeho schopnosti množiť sa v bunkách s aktivovanou *Ras* signálnou dráhou priniesol mnoho poznatkov o molekulárnom mechanizme účinku tohto vírusu v transformovaných bunkách a jeho schopnosti šíriť sa nádorovým tkanivom. Niektoré kľúčové otázky však ostávajú nezodpovedané. Je reovírus schopný navodiť antitumorálnu imunitnú odpoveď organizmu? Je jeho protinádorový účinok trvalý? Pôsobí rovnako intenzívne v hypoxickom nádorovom tkanive ako v normoxickom systéme nastavenom *in vitro*? Ako zvýšiť jeho účinnosť? K zodpovedaniu niektorých týchto otázok sme sa pokúsili prispieť našou prácou.

Naša imunizačná *in vivo* štúdia s reovírusom infikovanými bunkami priniesla neočakávané výsledky. Pri čelenži viac agresívnym typom buniek TC-1, sa ukázali ožiarené bunky ako lepší imunogén, avšak pri línii MK16, ktorá je menej agresívna, reovírusom infikované TC-1 bunky prakticky úplne pozastavili rast nádorov. Dôvody týchto rozdielov nepoznáme, avšak predpokladáme, že je to podmienené vlastnosťami porovnávaných buniek TC-1, ktoré sú podstatne onkogeénejšie a rastú rýchlejšie. Zaujímavé by bolo preskúmať ich molekukárnu podstatu, pretože lepšie poznanie imunizačnej schopnosti reovírusu môže hrať dôležitú úlohu v jeho ďalšom použití ako protinádorového terapeutika.

V *in vitro* štúdii sme potvrdili schonosť reovírusu vyvolať smrť nádorových buniek v hypoxii i v normoxii. Toto poznanie otvára cestu ďalším experimentom v hypoxii bez nutnosti použitia inhibítorov HIF-1α, pretože je známe, že HIF-1α často bráni v replikácii vírusov (Pipiya T. et al., 2005). Ďalej sme sa pokúsili objasniť akými mechanizmami spôsobuje reovírus lýzu a následnú smrť svojho hostiteľa, ktorým je pre nás vysoko žiaduca cieľová nádorová bunka. Zistili sme, že spôsob bunkovej smrti ním vyvolanej sa líši v normoxii a hypoxii. Nepodarilo sa nám objasniť presné mechanizmy bunkovej smrti, ktorými reovírus bojuje v normoxii a hypoxii, avšak aj zistenie, že to nie je autofágia, ktorou bunky usmrcuje v hypoxii, je prínosom v skúmaní spôsobenej onkolýzy. Podporenie jeho vrodených schopností spôsobovať bunkovú smrť svojho hostiteľa inými pro-apoptotickými terapeutikami určite prinesie nové úspechy v jeho použití na rôznych druhoch hypoxických nádorových buniek.

Ako ďalší logický krok vo výskume onkolytických vírusov vrátane reovírusu sa veľmi dôležitým javí skúmanie prepojenia ich účinku a aberantných pochodov v nádorovej bunke. Pochopenie určujúcich činiteľov transformácie dovoľuje idedntifikáciu a/alebo zostrojenie

nových účinnejších onkolytických vírusov. A naopak, výskum mechanizmov selektívnej vírusovej onkolýzy pomáha vylúštiť komplexné bunkové dráhy zahrnuté v bunkovej transformácii, identifikovať nové ciele protinádorovej terapie a navrhuje, ktoré nádorové pozadie je kompatibilné s virálnymi onkolytickými stratégiami. Výsledok virálnej infekcie úplne závisí od bunkových procesov. Preto vírusy s vrodenou alebo metódami genetického inžinierstva vloženou preferenciou k podmienkam nastaveným v nádorovej bunke poskytujú silný nástroj pre selektívny boj s nádormi. Nespočetné bunkové zmeny sú potrebné pre vznik tumorigenézy. Kľúč pre vývoj a použitie vírusov ako onkolytických agens je preto porozumenie unikátnych podmienok v transformovaných bunkách, ktoré sú pre replikáciu vírusov výhodné. Rozsiahly pokrok bol urobený na poli skúmania mechanizmov selektívnej virálnej onkolýzy. Diverzita bunkových a vírusových faktorov, ktoré dopomáhajú podmienkam vírusovej replikácie v transformovaných bunkách je veľmi pútavá.

Zatiaľčo status p53 dráhy a jadrový export vírusovej mRNA bol asociovaný s postačujúcou replikáciou E1B-55K deletovaného adenovírusu (ONYX-015), ďalšie rekombinantné adenovírusy obsahujúce delécie v E1A ukázali preferenciu k replikáci v nádorových bunkách s aberanciami v Rb proteíne (Heise C. et al., 2000, McCormick F., 2000, O'Shea C.C. et al., 2004). Selektívna replikácia onkolytického vírusu osýpok bola nedávno asociovaná so zvýšenou hustotou receptoru CD46 na nádorových bunkách (Anderson B.D. et al., 2004). Zároveň, aktivované Ras signálne kaskády a/alebo dysregulované dráhy translácie sú zahrnuté v selektívnej onkolýze viacerých vírusov spomenutých v úvode, z čoho zrejme vyplýva dôležitá úloha týchto procesov vo virálnej replikácii a bunkovej transformácii. Prečo je dysregulovaná translácia spoločnou perspektívnou témou vo virálnej onkolýze? Kontrola proteosyntézy je hlavný bunkový proces, ktorý je transformáciou zmenený. Zmeny v regulácii translácie nie sú jedine dôsledkom transformácie, ale môžu takisto spôsobiť tumorigenézu. Preto nie je prekvapivé, že antivírusové nechanizmy vedú k blokovaniu vírusovej proteosyntézy v normálnej bunke, a že v transformovanej bunke dysregulovaná translácia a kompromitované antivirálne mechanizmy sa spolupodieľajú na úspešnej virálnej replikácii (Shmulevitz M. et al., 2005). Najbližší výskum načrtne špecifické mechanizmy, ktorými reovírus využíva zlyhávajúce bunkové procesy na účinnú replikáciu. Skúmanie reovirálnej onkolýzy môže takisto odhaliť ďalšie spoločné znyky s ostatnými onkolytickými vírusmi.

Záverom možno zhrnúť, že sme potvrdili schponosť reovírusu infikovať a lyzovať nádorové ale i nenádorové bunky a to i v hypoxii. V exerimentoch *in vivo* sme preukázali schopnosť indukovať protinádorovú imunitu. Tieto poznatky môžu prispieť v ďalšom teoretickom výskume tohoto vírusu a takisto by mali byť zohľadnené pri jeho zavádzaní do klinickej praxe.

7. Prehľad publikácií, abstraktov a prednášok

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